UNIVERSIDADE DO ALGARVE

FACULDADE DE ENGENHARIA E RECURSOS NATURAIS

"Growth conditions, omega -3 fatty acid concentrations and gene expression of fatty acid desaturases in *Portulaca oleracea* leaves"

Condições de crescimento, concentração de ácidos gordos ómega-3 e expressão dos genes de desaturases em folhas de *Portulaca oleracea*

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Doctoral Thesis

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Mónica Correia Teixeira

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To my family and friends

Resumo

A beldroega *(Portulaca oleracea)*, foi seleccionada como sistema de estudo para este trabalho com base no seu elevado valor nutricional, em particular no conteúdo en ácidos gordos polinsaturados, e a tolerância demonstrada à seca e salinidade.

O trabalho de investigação aqui desenvolvido pode ser dividido em quatro pontos essenciais: (i) o estudo dos efeitos da salinidade no valor nutricional da beldroega; (ii) isolamento e caracterização dos genes de desaturases envolvidos na síntese de ácidos gordos polinsaturados; (iii) estudo do efeito de factores ambientais, como temperatura, salinidade e corte, nos níveis de expressão dos genes de desaturases e na accumulação de ácidos gordos polinsaturados; (iv) estabelecimento de linhas de cultura de células de *P. oleracea* para a produção *in vitro* de ácidos gordos polinsaturados e outras substâncias biologicamente activas.

Na primeira parte do trabalho avaliámos o efeitos de quatro regimes salinos (0, 60, 120 e 240 mM NaCl) na composição química da fracção aérea da beldroega. A salinidade afectou o desenvolvimento e o rendimento das culturas de beldroega, apesar de uma redução significativa da fracção aérea da beldroega ser apenas visível no regime salino mais elevado. A composição nutritional da beldroega foi negativamente afectada pela exposição aos tratamentos, com uma redução do potencial nutricional da cultura. Porém, a composição nutricional da cultura exposta a 60 mM NaCl foi similar à cultura control com um teor em lípidos totais mais elevado. As culturas de beldroega possuiam um elevado teor em minerais, com quantidades particularmente elevadas de magnésio e potássio. Quando expostas a regimes salinos elevados, os niveis de potássio e cálcio diminuiram e os niveis de sódio e magnésio aumentaram. Este desiquilíbrio iónico deu origem a elevados índices de Na/K, Mg/K, Na/Ca e Mg/Ca, o que resultou na redução de rendimento nas culturas expostas ao regime salino mais severo. De acordo com estes resultados podemos afirmar que a beldroega é uma excelente candidata à cultura em áreas de salinidade moderada ($\leq$ 60 mM) em que a introsão de água salgada ou a qualidade de água de irrigação constituem um problema para o estabelecimento de outras culturas mais sensíveis.

Uma vez que uma das características nutricinais mais relevante da beldroega é o seu elevado conteúdo em àcidos gordos polinsaturados, a segunda parte deste trabalho incidiu no estudo da biosíntese de ácidos gordos polinsaturados, particularmente no isolamento e caracterização dos genes que codificam para desaturases. Foram isolados três genes completos de omega-6 dessaturases, aqui designados por *PoleFAD2*-1, *PoleFAD2*-2 and *PoleFAD6*, e dois genes completos de omega-3 desaturases, aqui designados por *PoleFAD7* e *PoleFAD8*. Um clone parcial de uma potencial omega-3 desaturase microssomal, aqui designado por *PoleFAD3*-A foi também isolado. A sequência polipeptídica dos genes isolados apresentou elevada homologia com sequências já publicadas de outras espécies vegetais (GeneBank). Uma análise mais promenorizada das sequências polipeptídicas revelou a presenca de oito histidinas agrupadas em três motivos conservados na família das desaturases, essenciais para o estabelecimento do centro catalítico da enzima. A presença de um péptido sinal na extremidade azotada da cadeia, envolvido no transporte para o plastídio, nos clones *PoleFAD6*, *PoleFAD7* e *PoleFAD8*, indica uma elevada probabilidade destas enzimas estarem localizadas nas membranas dos cloroplastros. Nas duas isoformas do gene *FAD2* foi identificada uma sequência rica em amino-ácidos aromáticos (-YGNKS/ -YNNTL) na extremidade carbonada da cadeia polipeptídica, que tem sido referenciada como péptido sinal no transporte para o retículo endoplasmático. A previsão de três a quarto domínios transmembranares nas sequências polipéptidas sugere que estas desaturases são transmembranares.

Neste trabalho também foi incluída a expressão de dois dos genes plastidiais isolados, *PoleFAD6* e *PoleFAD7* em *Saccharomyces cerevisiae,* para a caracterização funcional das desaturases. Infelizmente, não fomos capazes de concluir este estudo com sucesso, uma vez que não foi detectada qualquer actividade dos clones nas condições testadas. Porém foi possível verificar, através de ensaios de fluorescência, que ambas as enzimas foram sintetizadas e accumuladas no organello alvo da célula: as membranas celulares. Temperatura, pH e suplementação do meio com ferro aparentemente não afectaram os níveis de expressão das enzymas, apesar de se ter detectado uma fraca actividade da omega-6 desaturase (*PoleFAD6*) a baixos valores de pH (4-5), identificada pela presença do produto de desaturação, ácido linoleico, no cromatograma (GC)._ Aparentemente, as enzimas estão a ser sintetizadas pela levedura e são acumuladas no organelo alvo, mas não numa forma funcional. È possivel que erros de tradução ou na formação do centro catalítico estejam na origem da producão de uma forma não activa da enzima.

Estudos anteriores demonstraram que factores ambientais, como a salinidade, afectam o teor em ácidos gordos polinsaturados nas culturas de beldroega, pelo que decidimos verificar o efeito da salinidade, corte e temperaturas baixas nos níveis de expressão dos genes das desaturases nesta cultura. Diferenças nos padrões de expressão destes genes foram detectadas para os diferentes stresses impostos, o que sugere que estes genes possam ter diferentes mecanismos de regulação a nível transcriptional ou pós-transcripcional. A exposição das culturas a temperaturas baixas (5 ºC) afectou os níveis de transcrição dos genes microsomais, *PoleFAD2*-2 e *PoleFAD3*, mas não afectou de uma forma significativa os genes cloroplastidiais, com excepção do *PoleFAD8*, em que um aumento significativo na expressão foi detectado em resposta às baixas temperaturas. Um acréscimo paralelo nos níveis de ácido linoleico(LA) e ácido linolénico(ALA) também foi detectado nas culturas expostas às baixas temperaturas. A aplicação de dois cortes longitudinais em folhas de beldroega originou um acréscimo dos níveis de expressão dos genes plastidiais, *PoleFAd6*, *PoleFAD7* e *PoleFAD8*, acompanhado de um aumento da accumulação dos respectivos produtos de desaturação LA e ALA. De um modo geral, a exposição das culturas a diferentes regimes salinos, induziu um decréscimo nos níveis de transcrição das desaturases, com excepção do gene *FAD8*, que apresentou um aumento significativo nos níveis de expressão com a exposição das culturas a um nível de salinidade moderado (60 mM NaCl).

Aparentemente factores ambientais tais como baixas temperaturas e salinidade têm um papel nos mecanismos de regulação da transcrição do gene *FAD8*, ilustrado neste estudo pelo acréscimo significativo dos níveis de expressão em todos os tratamentos aplicados.

A última parte deste trabalho consistiu no desenvolvimento de linhas de cultura de células a partir de explantes de *P. oleracea*, para a produção *in vitro* de ácidos gordos polinsaturados e/ou outros compostos biologicamente activos. O processo completo de produção de *calli* a partir da semente levou cerca de 6 -8 semanas. Três estruturas principais foram obtidas a partir de explantes de beldroega cultivados em meio sólido com diferentes proporções de fitohormonas (NAA/BAP): *calli* friáveis brancos (não fotossintéticos) / verdes (fotosintéticos) (WFC/ GFC), *calli* compacto verde com forma semelhante a um embrião (GCEC) e eflorescências de folhas vermelhas (RLE). Uma vez que algumas desaturases estam localizadas nas membranas dos cloroplastros, decidimos utilizar os *calli* fotosintéticos para estes estudos. Em cultura líquida, os *calli* friáveis originam uma estrutura circular parcialmente differenciada que pode ser utilizada para a produção *in vitro* de PUFAs. Quantidades mínimas de 3 mg LA/g tecido fresco e de 1 mg ALA/g fresco foram produzidas pelo sistema actual. Devido ao elevado índice de crescimento, com elevada produção de biomassa e o conteúdo em PUFAs, estas estruturas estão a ser avaliadas como potenciais fontes vegetais de PUFAs. Os resultados deste estudo originaram uma patente que está neste momento sob avaliação. Existe algum interesse na comercialização deste produto como suplemento nutricional para peixes e gado, com o objectivo de aumentar o conteúdo em ácidos gordos polinsaturados no músculo e produtos derivados animais.

Abstract

Purslane *(Portulaca oleracea)*, was selected as the plant system to be studied in this work due its high nutritional value, particularly in polyunsaturated fatty acids, together with its tolerance to drought and salinity.

This project was divided in four main areas of research: (i) study the effect of salinity in the chemical composition of *P. oleracea* L. shoots; (ii) the isolation and characterization of fatty acid desaturase genes from *P. oleracea* L., involved in PUFAs biosynthesis; (iii) Study the effect of environmental stresses in the expression of desaturase genes and PUFAs accumulation in purslane leaves; (iv); and the establishment of cell culture lines of *P. oleracea* for the *in vitro* production of PUFAs and other molecules biologically active.

For the first part of this project we tested the effect of four chloride-salinity regimes (0, 60, 120 and 240 mM NaCl) in purslane shoots chemical composition. The plant were cultured in a green-house and exposed to the treatments for 15 days. We analyzed culture yield, proximal nutritional composition and mineral composition after 7 and 15 days of treatment. Salinity affected the growth and yield of purslane plants, although a yield reduction was only observed in plants exposed to the highest level of salinity. Salinity also significantly affected the proximal nutritional composition of purslane leaves with a decrease in potential nutritional value; however, purslane plants exposed to moderate salinity conditions, 60 mM NaCl, showed a chemical composition similar to the control plants and higher oil content. This study also showed that purslane has a high mineral content; particularly high amounts of magnesium (Mg) and potassium (K). When exposed to elevated salinity levels, potassium and calcium levels decreased and sodium and magnesium levels increased. This led to a significant increase in the Na/K, Mg/K, Na/Ca and Mg/Ca ratios and was reflected in the reduction of the growth and yield of purslane plants exposed to the more extreme salinity treatment.

According to this study, purslane appears particularly well suited for cropping in areas whereas sea water intrusions or poor irrigation water quality constitute a problem to the establishment of more sensitive crops.

Due to purslane characteristic high polyunsaturated fatty acid content, the second part of this project was focused in the isolation and characterization of the genes associated to the fatty acid metabolism, more specifically the genes encoding for fatty acid desaturases. Here, we were able to isolate three complete cDNAs that coded for putative omega-6 desaturase genes, hereby designated by *PoleFAD2*-1, *PoleFAD2*-2 and *PoleFAD6*, and two complete cDNAs that coded for putative omega-3 desaturase genes, hereby designated by *PoleFAD7* and *PoleFAD8*. A partial fragment of a putative microsomal cDNA hereby designated and *PoleFAD3*-A was also isolated. Sequence analysis showed high similarity of the isolated genes with published sequences from other plant species (Gene Bank). A more detailed analysis revealed the presence of eight histidine in three separate clusters that constitute conservative motifs found in all membrane bound desaturases essential for the establishment of the catalytic site The presence of putative N-terminus transit peptide involved in the transport into the plasmid, in *PoleFAD6*, *PoleFAD7* and *PoleFAD8* genes, indicated a high probability of these desaturases being localized in the chloroplast membranes. Both isoforms of *PoleFAD2* amino acid sequence have an aromatic amino acid-enriched sequence at the C-terminus (-YGNKS/ -YNNTL) that has been found to act as an ER retrieval signal. The prediction of three to four putative transmembrane in the encoded polypeptides also support the notion that these desaturases are membrane bound.

In this project we also attempt to express two plastid genes *PoleFAD6* and *PoleFAD7* in *Saccharomyces cerevisiae* cells in order to do a functional characterization of the desaturase enzymes. Unfortunally we were not able to do so in a successful way, since no activity was detected for either of the enzymes under the studied conditions. However we were able to access, by fluorescence studies, that both enzymes were synthesized and accumulate preferentially in the target cell organelle: cell membranes. Growth temperature, pH and iron supplementation on the medium did not seem to affect the desaturase heterologous expression in yeast, although a detectable activity of the enzyme coded by *PoleFAD6* was present at pH values of 4 to 5 units, revealed by the presence of the desaturation product, LA, in the GC chromatogram. Apparently the heterologous FADs were synthesized by the yeast cell and accumulated in the right cell organelle, but in an inactive form. Error in translation or on the protein structure may affect the catalytic site, and lead to the formation of inactive/low active forms of the enzymes.

Previous studies had revealed that environmental growth conditions affect the PUFAs content in purslane shoots, so for the third part of this project we analyzed the effect of chilling temperatures (5 ºC), salinity and wounding on the transcript accumulation of these desaturase genes in this culture. Differences in expression pattern were detected for the different treatments which suggest that some of these genes have different transcriptional and/or post-transcriptional regulation mechanisms. Low temperatures lead to an increase in transcript levels of the microsomal desaturases genes, *PoleFAD2* and *PoleFAD3*, but had a minor effect on the chloroplastidial genes, with the exception of *PoleFAD8* that showed a significant increase in expression as a response to low temperature exposure. An increase on LA and ALA levels was also detected in the leaves of the stressed plants. On the other hand, wounding induced a significant increase in the transcript levels of chloroplastidial genes, *PoleFAD6*, *PoleFAD7* and *PoleFAD8*, followed by an increase on the accumulation of the desaturation products, LA and LNA, in purslane leaves. Microsomal genes transcript levels were not significantly affected by wounding, although a slight increase of expression was detected. Environmental salinity negatively affected the transcription of desaturase genes, leading to lower levels of expression, with the exception of FAD8 gene, that showed an increase in expression in cultures exposed to moderated levels of salinity (60 mM NaCl).

Apparently environmental factors have a direct role in the regulation/activation mechanisms of FAD8 transcription, revealed in this study by an increase in the transcript levels in all applied treatments.

Finally, the last part of this project aim to the establishment of *P. oleracea* cell culture lines for the in vitro production of PUFAs and other compounds biologically active. The full processes from seeds to the development of *calli* took 6-8 weeks. Three main structures were formed in solid media supplemented with different proportions of growth hormones (NAA/BAP): soft white (non-photosynthetic)/green (Photosynthetic) friable *calli* (WFC/GFC), compact green embryo-like *calli* (CGEC) and red leafs efflorescence (RLE). Since same desaturases are known to be localized in the chloroplast membranes, we decided to use the photosynthetic friable calli (GFC) for these studies. GFC formed a semi-differentiated oval structure in liquid culture that can be used for the in vitro production of PUFAs. At least 3 mg LA/g fresh tissue and 1 mg ALA/g fresh tissue were produced by the current developed system. Because of the observed high growth rate, leading to good biomass production and its PUFAs content, these structures are under evaluation as good sources of plant omega-3. The results of these studies originated a patent application that is now under evaluation. Some interest has been shown to use these structures as food supplement for fish and livestock to increase PUFAs content in the muscles and animal by products.

List of Publications

Paper I: Monica C. Teixeira and Isabel S. Carvalho (2008). Effects of salt stress on purslane (*Portulaca oleracea*) nutrition. Annals of Applied Biology, 154 (1), 77-86.

Paper II: Molecular Cloning and expression analysis of three omega-6 desaturase genes from purslane (*Portulaca oleracea* L.) (In press, Biotechnology Letters)

Paper III: Omega-3 fatty acid desaturase genes isolated from purslane (*Portulaca oleracea* L): Expression in different tissues and response to cold and wound stress. (Submitted: article ID: jf-2009-009015)

Paper IV: Differential expression of omega-3 and omega-6 desaturase genes and linolenic/linoleic accumulation levels in leaves of *Portulaca oleracea* L., exposed to chloride-salinity treatments. (Submitted: article ID: Planta-2009-03-0151)

Paper V: Establishment of in vitro plants, cell and tissue cultures from *Portulaca oleracea* for the production of polyunsaturated fatty acids (Manuscript)

Abbreviations

FAD- Fatty acid desaturase
ER- Endoplasmatic reticulum
TFA- Total Fatty acids
PUFA – Polyunsaturated fatty acid
DA - Dienoic acids
TA – Trienoic acids
FAME – Fatty acid methyl esters
PCR –Polymerase chain reaction
RACE- Rapid Amplification of cDNA Ends
RT-qPCR – Real time semi-quantitative PCR
RT-PCR – Reverse transcriptase PCR
TOA – Total oxalic acid
MS – Murashi and Skoog medium
WFC- White friable callus
GFC – Green friable callus
GCEC – Green compact embryo-like callus
GCC – Green compact callus
RLE – Red leaf efflorescence

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Appendice 1 - Paper I: Monica C. Teixeira and Isabel S. Carvalho (2008). Effects of salt stress on purslane (*Portulaca oleracea*) nutrition. Annals of Applied Biology, 154 (1), 77-86.

1. Literature Review

1.1. Purslane: nutritional crop or infesting weed?

Portulaca oleracea (*Portulacaceae* family) is listed in the World Health Organization as one of the most used medicinal plants and it has been given the term "Global Panacea" (Dweck, 2001; Samy *et al*.., 2004). It is available commercially in both ornamental and culinary cultivars. Recent research has showed that the *P. oleracea* varieties, particularly the ornamental cultivars, have high total phenol content and exhibit antioxidant activity (Lim and Quah, 2007). Common purslane (*Portulaca oleracea* L.), is a member of the Portulacaceae, which consist of more than 120 species of often succulent herbs and shrubs. It is a very versatile plant that has been rated as moderately tolerant to salinity and draught (Kumamoto *et al*., 1990; Grieve and Suarez, 1997; Maas and Grattan, 1999), which enables it to colonize all kinds of soils (Kamil *et al*., 2000; Matthews *et al*., 1993).

Purslane has a relative short life cycle, in favorable conditions this plant completes its life cycle in only 60 days (Fig. 1.1.1). The seeds germinate in large number on the soil surface after irrigation or rain, when soil temperature reaches 15ºC (60ºF); seedlings grow fast and reach the adult state in 4 to 6 weeks, after which flowering occurs and seed production initiates shortly thereafter. The mature seeds are released 14 to 16 days after flowering (Rashed *et al*., 2003; Matthew *et al*., 1993; Kedshen, 1987) it is fast growing, self-compatible and produces large number of seeds that have a long viability.

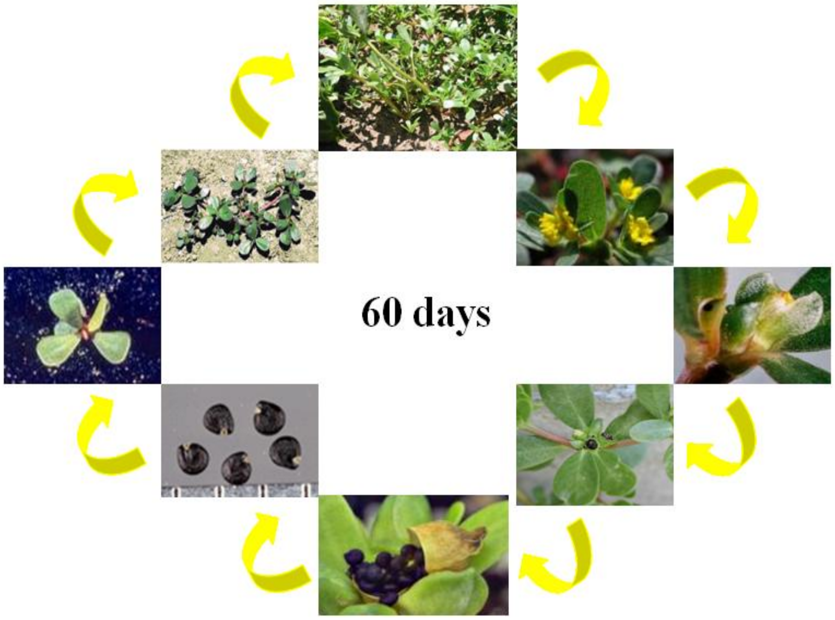


Figure 1.1.1. Purslane (*Portulaca oleracea L.*) life cycle

These days, most people in modern societies limit their diet to a few cultivated vegetables so that wild plants such as purslane tend to be under-utilized. Recent research indicates that purslane offers better nourishment than the major cultivated vegetables. The stems and the leaves of the plant are succulent and edible with a slightly acidic and salty taste similar to spinach. A nutritive characterization of purslane accessions conducted by Ezekwe *et al.* (1999) showed that, in spite of its genetic diversity, purslane remains one of the most abundant terrestrial vegetable sources of omega-3 fatty acids and other essential nutrients potentially beneficial for humans as well as animals. Purslane shoot is a rich source of omega-3 fatty acids (Kumamoto *et al.*, 1990; Omara-Alwala *et al.*, 1991; Simopoulos, 2004; Palaniswany *et al.,* 2001), α-tocopherols, ascorbic acid, β-carotene, and glutathione (Simopoulos *et al.*., 1992). Other constituents of purslane include, calcium oxalate, malic and citric acids, dopamine and dopa, coumarins, flavonoids, alkaloids, saponins(Iwu. 1993), and urea, among others (Leung and Foster, 1996). It is also rich in minerals such as potassium (Dweck, 2001), magnesium (Keys, 1976), calcium and iron (Burkill 1995).

Total fatty acid content varies among purslane tissues. It was higher in seeds and leaves (Guil *et al.*, 1996; Liu *et al.,* 2000; Simopoulos, 1992), and lowers in stems (about one third). The presence and concentration of α-linolenic acid in purslane may vary with the cultivar, geographic distribution, developmental stage and environmental factors (Liu *et al.*., 2000, 2002; Ezekwe *et al.*., 1999; Palaniswamy *et al.*, 2001) The content of α-linolenic acid (ALA) in purslane also varies with the age and tissue-type, with leaves containing higher concentrations then the stems (Simopoulos *et al.*., 1992). ALA accounted for 60% of TFA in leaves, 30-40% in seeds and 10 to 25% in stems (Guil *et al.*, 1996; Liu *et al.,* 2000). Except for linseed, in which ALA is 50-60% of the total fatty acids, most other oil crop seeds contain only 3 to 10 % ALA. So purslane constitutes an excellent vegetable source of ALA.

P. oleracea is widely used as a potherb in the Mediterranean, Central European and Asian countries. The high content in omega-3 fatty acids of *P. oleracea* is an important factor in the prevention of heart attacks and strengthening of the immune system (Simopoulos, 2004). The aerial parts are used medicinally for alleviating pain and swelling and as an antiseptic (Chan *et al.*., 2000). The dried herb can be boiled and it is made into a tea/soup in China (Cai *et al.*., 2004).This plant was reported to have neuropharmacological actions, wound healing activities and bronchodilatory effects (Malek, *et al.*., 2004; Parry *et al.*., 1993; Rashed *et al.*., 2003). The water extracts of *P. oleracea* show no cytotoxicity or genotoxicity, and have been certified safe for daily consumption as a vegetable (Yen *et al.*, 2001). The methanol extracts from this plant were found to exhibit moderate antimicrobial activity against *Bacillus subtiliis* (Sakai, *et al.*, 1996). The inhibitory effect on lipopolysaccharide (LPS) and interferon-γ (IFN-γ)-induced nitric oxide (NO) production was shown by the extracts of *P. oleracea* in a concentration dependent manner (Abas *et al.*, 2006).

Due to its high content of nutrients, especially antioxidants and omega-3 fatty acids, and its wound healing and antimicrobial effects as well as its traditional use in topical treatment of inflammatory conditions, purslane is a highly likely candidate as a useful cosmetic ingredient (Leung and Foster, 1996).

1.2. Fatty Acid Biosynthesis in plants

In plants, lipids play important roles in energy storage, membrane structure, biological activity, and surface coverings, in addition to some specialized functions such as light harvesting during photosynthesis. Plants are fundamentally different from other eukaryotes in the molecular organization of the enzymes of fatty acid synthesis. The predominant plant fatty acids found in nature consist of just six or seven structures that have chain lengths of 16 or 18 carbons and one to three double bonds. These fatty acids are synthesized by the enzymes acetyl-CoA carboxylase

(ACCase) and fatty acid synthase (FAS) from acetyl CoA. The central carbon donor for fatty acid synthesis in the malonyl-CoA produced by ACCase. In the initial reactions, desaturases act on the preformed long-chain acyl esters, with O_2 and a reduced compound such as ferrodoxin or cytocrome b_5, as cofactors. The assembly of fatty acids and the introduction of the first double bond occurs while these structures are attached to protein cofactor, acyl carrier protein (ACP). From this point on, all the reactions of the pathway involve ACP until the 16- or 18-carbon product is ready for transfer to glycerolipids or export from the plastid (Ohlrogge and Browse, 1995).

At least three separate condensing enzymes (3-ketoacyl-ACP synthases – KAS) are required to produce an 18-carbonfatty acid. KAS III catalyzes the first condensation of acetyl-CoA and malonyl-ACP to form a 4-carbon product. KAS I produces chain lengths from six to sixteen carbons. The elongation of the 16-carbon palmitoyl-ACP to steoryl-ACP is catalyzed by KAS II. The initial product of each condensation is a 3-ketoacyl-ACP. This is followed by three additional reactions (reduction, dehydration, reduction) to form a saturated fatty acid. After each round of four reactions, the precursor fatty acid is lengthened by two carbons. The elongation of fatty acids in the plastids is terminated when the acyl group is removed from ACP.

Substrate selectivity of acyl-ACP thioesterases dictates that palmitate and stearate are the two products formed. The major fate of 16:0 and 18:0 acyl chains produced in the plastid is to form the hydrophobic portion of glycerolipids molecules which are components of all cellular membranes. Subsequent desaturation of these lipids to the highly unsaturated forms typical of plant cell membranes is carried out by membrane-bound desaturases of the chloroplast and the endoplasmic reticulum (ER) (Browse and Somerville, 1991; Ohlrogge and Browse 1995). The main substrate for the Δ12 desaturase is 1-acyl, 2-oleoyl-phosphatidylcholine in the endoplasmic reticulum of the cell (although other lipids may also be substrates in chloroplasts). The newly formed linoleate is then transferred by a variety of mechanisms to other lipids.

Oil producing crop plants synthesize and storage energy in the form of triacylglycerols (TAG), which are composed of a glycerol backbone molecule esterified by three saturated and/or unsaturated fatty acyl groups. Unsaturated fatty acids are essential components required for normal cellular function, being involved in roles ranging from membrane fluidity to acting as signal molecules (Gil and Valivety, 1997; Broun *et al.*, 1999). In particular, the class of fatty acids known as the polyunsaturated fatty acid*s* (PUFAs) has attracted considerable interest as pharmaceutical and nutraceutical compounds (Broun *et al.*, 1999; Horrobin, 1990). PUFAs can be defined as fatty acids of 18 carbons or more in length, containing two or more double bonds. These double bonds are inserted by specific fatty acid desaturase enzymes that have been the subject of intense research in recent years (Shanklin and Cahoon, 1998; Napier *et al.*, 1999). Linoleic and α-linolenic acids are synthesized in plant tissues from oleic acid by the introduction of double bonds between the existing double bond in the ninth position, and the terminal methyl group by the sequential action of Δ12 and Δ15 desaturases (Fig. 1.2.1.).

$$\underset{n\text{-}9}{18{:}1^{\Delta 9}} \xrightarrow{\Delta\text{-12 Desaturase}} \underset{n\text{-}6}{18{:}2^{\Delta 9,12}} \xrightarrow{\Delta\text{-15 Desaturase}} \underset{n\text{-}3}{18{:}2^{\Delta 9,12,15}}$$

Figure 1.2.1. Desaturation reaction. Sequential conversion of oleic acid into α-linolenic acid by the combined action of a Δ12- and a Δ15 fatty acid desaturases.

Essential fatty acids are required in the diet for normal development in mammals that cannot synthesize the primary essential fatty acid-PUFA, linoleic acid (Broun *et al.*, 1999 ;). The PUFAs can be classified into two groups, *n-6* or *n-3*, depending on the position (*n*) of the double bond nearest the methyl end of the fatty acid (Gill and Valivety, 1999; Broun *et al.*, 1999; Napier *et al.*, 1999). Thus, γ-linolenic acid ($18{:}3\Delta^{6,\ 9,\ 12}$) is classified as an 18:3, *n-6* PUFA, whereas α-linolenic acid ($18{:}3\Delta^{9,\ 12,\ 15}$) is an 18:3, *n-3* PUFA. Both ω-3 and ω-6 polyunsaturated fatty acids (PUFA) are important as structural components of membrane glycerolipids and as precursors to signaling molecules such as jasmonates in plants and eicosanoids in animals (Heinz, 1993; Hamada *et al.*, 1996).

1.2.1. Fatty Acid Desaturases (FADs)

Fatty acid desaturases are enzymes found in microbes, plants and animal cells and are essential in a number of physiological processes. They are responsible for the insertion of double bonds (normally in the Z or cis conformation) into alkyl chains, following the abstraction of two hydrogen atoms. Plant desaturases fall into two categories: soluble and membrane-bound. Membrane-bound desaturases are located in both ER and chloroplast membranes while soluble plant desaturases are localized in the chloroplast stroma. In figure 1.2.1.1.-A is represented the Crystal structure of a soluble Δ-9 stearoyl-acyl carrier protein desaturase isolated from Castor seed crystal in the form of a dimmer. Each monomer is composed by α-helices, with a N- and C-terminal domains, and three histidine boxes where the di-iron center connected to 4 of the α-helices (Lindqvist, *et al.*, 1996). There is considerable sequence conservation among the membrane-bound desaturases (Shanklin *et al.*. 1995). Each individual desaturase tends to be specific in the chain length and the double bond insertion position of its fatty acid substrate. In figure 1.2.1.1.-B is represented a putative arrangement of membrane bound desaturases, with N-terminal and C-terminal domains, the 3 characteristic histidine boxes domain were the iron (Fe) connected to form the catalytic site and 4 trans-membrane helices.

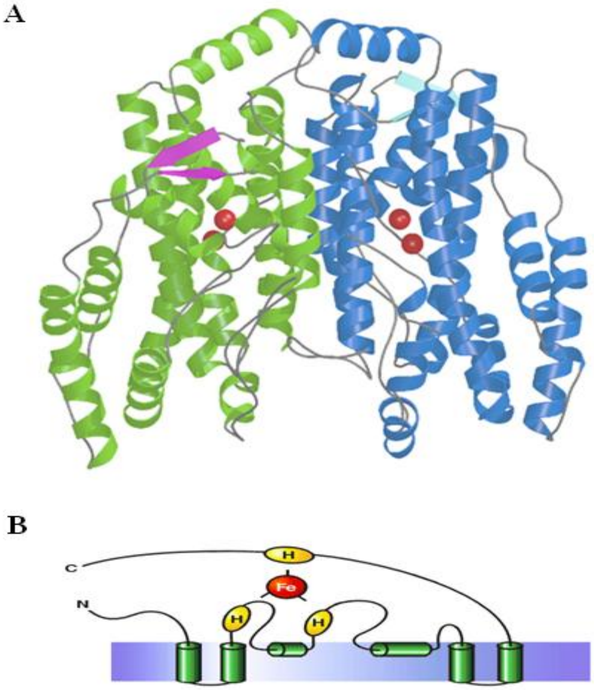


Figure 1.2.1.1. Structure of fatty acid desaturases. (A) Model of the crystal structure of delta-9 stearoyl-acyl carrier protein desaturase from Castor seed in the form of a dimmer. Each monomer is composed by α-helices, with a N- and C- terminal domains, three histidine boxes where the di-iron center (red spheres) connected to 4 of the α-helices (1st – E196 and H232; 2nd – E143 and E229) (Lindqvist, *et al.*, 1996). (B) Putative arrangement of membrane bound desaturases, with N-terminal and C-terminal domains, the 3 characteristic histidine boxes (H) domain were the iron (Fe) connected to form the catalytic site and the 4 trans-membrane helices are represented.

In plants, the microsomal ω-6 desaturase catalyzed pathway is the primary route of polyunsaturated lipid production. This membrane-bound enzyme catalyzes the first extra-plastid desaturation in plants and converts oleic acid esterified to phosphatidylcholine to linoleic acid.

The regulation mechanism of the chloroplast and ER desaturases has been study in *Arabidopsis thaliana* mutants, each deficient in a specific desaturation step (Fig. 1.2.1.2).Mutations in two loci, *FAD2* and *FAD3*, primarily affected desaturation of the extra chloroplast lipids, whereas mutations in the remaining five loci, *FAD4, FAD5, FAD6, FAD7* and *FAD8*, affected chloroplast lipid desaturation (Fig. 1.2.1.2.)(Ohlrogge and Browse, 1995).

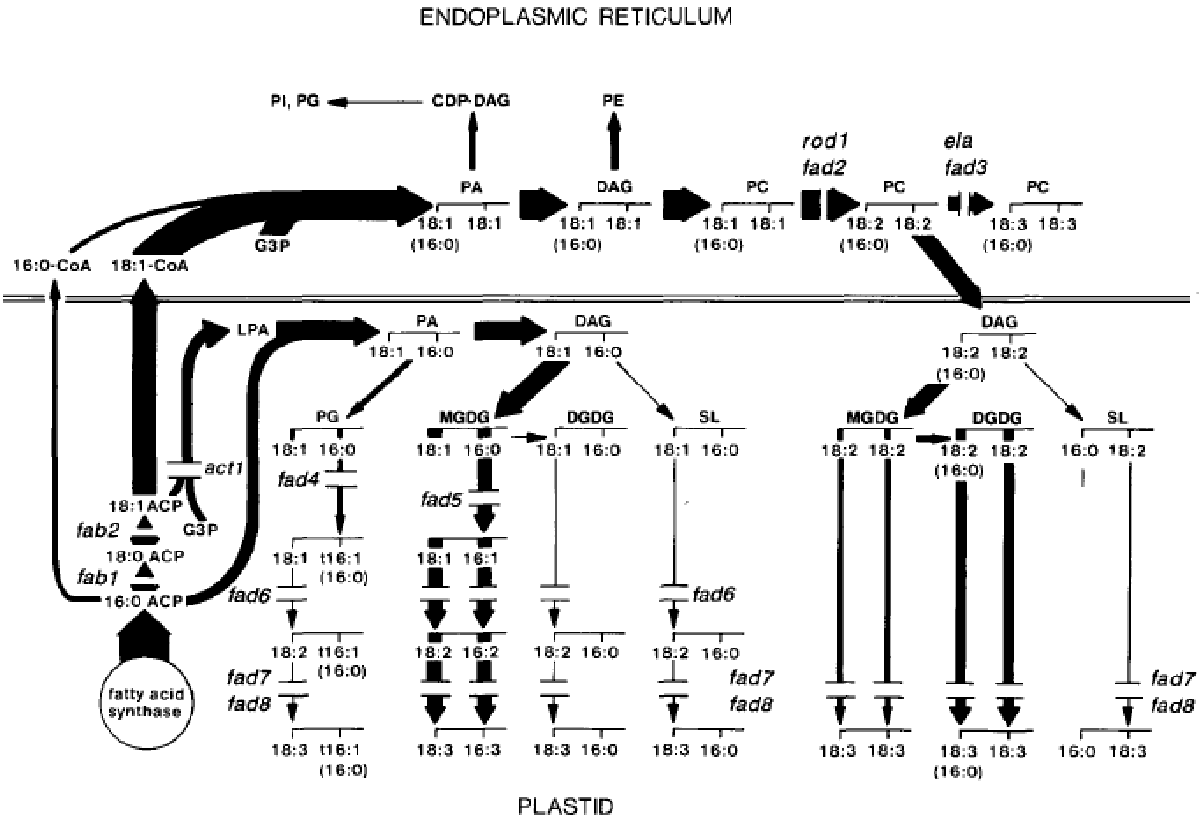


Figure 1.2.1.2. An abbreviated diagram of fatty acid synthesis and glycerolipid assembly in *Arabidopsis* Leaves (adapted from Ohlrogge and Browse (1995). Widths of the lines show the relative fluxes through different reactions. The breaks indicate the putative enzyme deficiencies in various mutants. ***ela***, enhanced linoleate accumulation; ***mdl,*** reduced oleate desaturation.

Omega-3/6 fatty acid desaturases seem to be deeply embedded within biomembranes owing to the difficulty of characterizing it by conventional biochemical approaches, so its gene has been cloned using genetic techniques, namely map-based cloning methods that used mutant strains of *Arabidopsis* (Arondel *et al.*, 1992). Two distinct desaturases have been characterized that can insert the Δ12 double bond, i.e. an ω-6 desaturase plastid enzyme (*FAD6*), which uses the terminal methyl group as a reference point, and secondly an extra-plastid oleate Δ12 desaturase (*FAD2*) that is selective for C-12, 13 oxidation independently of chain length (Fig. 1.2.1.2.; Miquel and Browse, 1992; Gibson et al., 1994; Ohlrogge and Browse 1995). *FAD2* genes showing tissue-specific expression have been identified in cotton (Liu *et al.*. 1997), sunflower (Martinez-Rivas *et al.*., 2001), *Arabidopsis thaliana* (Okuley *et al.*., 1994), soybean (Heppard *et al.*, 1996), parsley (Kirsch and Hahlbrock, 1997), rape (Scheffler *et al.*,1997), peanut (Jung *et al.*, 2000), sesame (Jin *et al.*, 2001), sunflower (Martinez-Rivez, 2001), cotton (Pirtle, 2001), tung (Dyer, 2002), spinach (Saeki *et al.*, 2004) and olive (Hernandez *et al.*, 2005). cDNAs encoding for plastid ω-6 desaturases have also been isolated from different species including rape (Hitz *et al.*, 1994), soybean (Hitz *et al.*, 1994), and spinach (Murphy and Piffanelli, 1998).

Omega-3 fatty acid desaturases catalyzes the insertion of a third double bound into linoleic acid precursors to produce linolenic acid. The omega-3 fatty acid desaturase genes cloned thus far are divided in two types, one localized in the chloroplasts (*FAD7* and *FAD8*), which uses the terminal methyl group as a reference point (Gibson *et al.*, 1994; Hugly *et al.*, 1989)) and the other localized in the ER (*FAD3*) (Arondel *et al.*, 1992, Ohlrogge and Browse, 1995) (Fig. 1.2.3.). Seed linolenic acid levels have been shown to be determined by microsomal ω-3 desaturase genes (Yadav *et al.*. 1993). *Arabidopsis thaliana* has one *FAD3* gene (Yadav *et al.*, 1993), while three independent microsomal ω-3 desaturase genes (*GmFAD3*A, *GmFAD3*B and *GmFAD3*C) have been identified in soybean (Bilyeu *et al.*, 2003). These three genes showed tissue-specific expression and the absence of *FAD3*A were linked to the low linolenic acid phenotype in the A5 soybean line. In another study (Vriten *et al.*., 2005), identified two *FAD3* desaturase genes (*FAD*3A and *FAD3B*) that control the level of linolenic acid in flax. Analysis showed that in a low-linolenic-acid line, both genes contained point mutations leading to premature stop codons and consequently inactive truncated protein. Genes encoding two plastid omega-3 desaturases (*FAD7/FAD8*) were previously isolated from *Glycine max* (Van de Loo and Somerville, 1994), *Arabidopsis thaliana* (Gibson *et al.*, 1994; Iba *et al.*, 1993), and *Zea maize* (Berberich *et al.*, 1998).

Fatty acid desaturases in all organisms are subjected to several types of regulation, depending on their localization or function, and there may be requirement for different or specific desaturase activities during certain development processes in various tissues (Murphy and Piffanelli, 1998). A More complete knowledge of fatty acid unsaturation, mobilization, and regulation processes may significantly aid the development of effective strategies for managing oil and essential fatty acids accumulation in plants.

1.3. Plant-environment interactions: abiotic stresses

Plants often encounter the abiotic stresses of low or elevated temperature, exposure to salt, drought, and, less commonly, heavy metals, as well as biotic pathogen and insect attack, sometimes simultaneously. For example, when a plant is subjected to abiotic environmental stresses, such as high or low temperatures, intense light, or drought, its sensitivity to biotic stresses such as viruses and bacteria could be also intensified (Gaudet *et al.*, 1999; Kuwabara *et al.*, 2001). Previous studies of plant resistance to temperature have regarded temperature extremes as the sole source of stress that affects populations under natural conditions, even if tolerance to drought and salt stresses or tolerance to freezing was also conferred on the plants (Kasuga *et al.*., 1999). For example, this addition would still keep the tolerance within only a single stress category because these stresses are mainly caused by a single effect of dehydration on cells. As a result, the development of a more effective approach to creating plants that can resist a wide range of stresses remains a challenge for the future. Even with the best available land and agricultural practices, the impact of abiotic stresses can significantly reduce the productivity of food and fiber crops.

Environmental salinity leads to a reduction of the plants ability to uptake and accumulate valuable nutrients, causing a nutrient imbalance that subsequently leads to the reduction of plant growth. Reports have been made that evidence the fact that plants have shown a decrease in culture yield when exposed to saline environments. D'Amico *et al.* (2003) reported a marked reduction in the yield of tomato cultures (*Lycopersicon esculentum* Mill.) exposed to saline levels of 8 dS m-1 and 14 dS m-1, respectively, with corresponding yields of 69 % and 43 % of the control (3 dS m-1). Lacerda *et al.* (2003) also reported a reduction in the yield of two forage sorghum genotypes (*Sorghum bicolor* L.) differing in salt tolerance, when exposed to salinity levels of 100 mM NaCl.

Not only the yield of the culture is affected by environmental salinity, but also the nutritional value of the crops is affected. High concentrations of sodium and chloride ions in soil or irrigation solutions may reduce the uptake and accumulation of ions and nutrients, leading to extreme ratios of Na/Ca, Na/K, Ca/Mg and Cl/NO3 (Grattan and Grieve, 1999). In high salinity conditions, high concentration of Na not only interferes in K uptake at root level but also causes alterations in the integrity and selectivity of membranes. Studies performed in a large variety of vegetables revealed a significant reduction of K levels in the plant tissues with the increase in Na and/or increase in Na/Ca ratio in the roots (Pérez-Alfocea *et al.*, 1993; Gorham, 1994; Grattan & Grieve, 1999).

In many plants, protein synthesis is affected by the exposure of the plant to sodium chloride, and in some cases, protein hydrolysis occurs with the release and accumulation of free amino acids in the tissues (Levit, 1980; Neumann, 1997; Nilsen & Orcutt, 2000). Furthermore, the increase in uptake and accumulation of chloride ions (Cl^-) in plant tissue generally results in the decrease in nitrate (NO_3^-) accumulation in the plant's aerial parts (Rieger and Litvin, 1998; Nilsen and Orcutt, 2000; Ottow *et al.*, 2005). Examples of this have been found in cucumber, *Cucumis sativus* L. (Martinez and Cerdá , 1989), eggplant, *Solanum melongena* L. (Savvas and Lenz, 1996), melon, *Cucumis melo* L. (Feigin *et al.*, 1987; Kafkafi *et al.*, 1992), tomato, *Lycopersicon esculentum* (Kafkafi *et al.*, 1992; Feigin *et al.*, 1987; Pérez-Alfocea *et al.*, 1993) and barley, *Hordeum vulgare* L. (Britto *et al.*, 2004). Many have attributed this reduction to Cl^- antagonism of NO_3^- uptake (Feigin *et al.*, 1987; Savvas and Lenz, 1996).

Many studies have reported that saline stress causes a reduction in total lipid accumulation in plant tissues t.ex. sugar beat (Stuiver *et al.*, 2006), cowpea (Vigna unguiculata; Vazquez-Duhalt *et al.*, 2004), sunflower seeds (Flagela *et al.*, 2004), and mustard seeds (*Brassica juncea L.;* Parti *et al.,* 2003). However in some isolated cases, such as purslane (Teixeira and Carvalho, 2008) and *Oenothera biennis* (Heuer *et al.*, 2002) an increase of total lipid content was registered in leafs and seeds, respectively, when the plant was subjected to moderated salinity level ($ECi \leq 6\ dSm^{-1}$).

The accumulation of organic compounds, such as sugars and amino acids, in the cytoplasm plays an important role in the osmotic balance of plants (Morgan, 1992). An increase in carbohydrates content as a response to saline treatment has also been measured in plants. Kafi *et al.* (2003) reported an increase in total carbohydrate content in leaves of wheat cultivars with increasing salt content in the medium (300 mM NaCl). Watanabe *et al.* (2000) reported an increase in the total sugar content of young leaves of *P. euphratica* for NaCl concentration of 250 mM, in an in vitro test.

Thus the current situation of diminishing farm land world wide and the potential heightened effects of global climate changes on environmental, pathogen and insect stresses provide impetus to understand stress resistance in crop plants.

1.3.1. Abiotic stress effects in cellular membrane structure and permeability

Since plants lack mobility they cannot avoid exposure to stresses in their environment, but most adapt to them in other ways. Plants have evolved both constitutive and inducible means to resist stresses (Upchurch, 2008).

Fatty acids are crucial components of cellular membranes, suberin, and cutin waxes that provide structural barriers to the environment (Beison *et al.*, 2007). They contribute to inducible stress resistance through the remodeling of membrane fluidity (Iba, 2002), the release, through lipase activity, of α-linolenic acid (Grechkin 1998), and as modulators of gene expression (Kachroo *et al.*, 2001), and seed colonization by fungi (Calvo *et al.*, 1999). Modification of membrane fluidity results in an environment suitable for the function of critical integral proteins, such as the photosynthetic machinery, during stresses. Free linoleic acid is itself a stress signal and the precursor for phyto-oxylipin biosynthesis (Blée 2002). The ability to adjust membrane lipid fluidity by changing levels of unsaturated fatty acids is a feature of stress acclimating plants provide mainly by the regulated activity of fatty acid desaturases. The expressions of each of the omega-3 fatty acid desaturase genes appear to be regulated in a complex way in response to changes in the environment or other stress inducing factors. For example, environmental stimuli, such as wounding, salt stress, and pathogen invasion, lead to a rapid increase in a defense-related signal molecule, jasmonate. Trienoic fatty acids (TAs), especially α-linolenic acid is a precursor of jasmonate (Nishiuchi and Iba, 1998; Farmer, 1994), and the stimulus leads to a rapid induction of the expression of the chloroplast omega-3 desaturase genes (*FAD7* and *FAD8*) (Hamada *et al.*., 1996; Nishiuchi *et al.*.,1997, 1999). In contrast, the expression of the ER omega-3 desaturase gene (*FAD3*) is regulated through the synergistic and antagonistic interaction of plant hormones such as auxins, cytokines, and abscisic acid, and the tissue specificity of the expression of this gene is further modified in accordance with the growth phase in plant development (Matsuda *et al.*., 2001; Yamamoto, 1994; Zou *et al.*., 1995).

In view of these facts, the regulation of fatty acid desaturation of membrane lipids appears to be intimately related to the wide range of mechanisms that allow plants to adapt to their environmental throughout development. More complete knowledge of fatty acid unsaturation, mobilization, and regulation processes may significantly aid the development of effective strategies for managing abiotic and biotic stresses for these plants (Upchurch 2008).

1.3.1.1. Temperature effect on membrane stability and FAD activity

The physiochemical characteristics displayed by lipid bilayers at different temperatures differ with the species of the lipid head group or their esterified fatty acids, and their lipid constituents and fatty acid constituents change depending on the environmental growth temperatures (McConn, *et al.*, 1994; Iba, 2002). At low temperatures plant membranes undergo transition from liquid crystalline to a gel-like phase with reduced fluidity and ion leakage and deactivation of membrane proteins occurs. Maintenance of polyunsaturated fatty acid levels in chloroplast lipids has been shown to contribute to low temperature survival and the normal formation of chloroplast membranes under chilling stress (Routaboul *et al.*, 2001; Iba 2002, Upchurch 2008). There is a general inverse relationship between polyunsaturated of fatty acids and growth temperature, polyunsaturated fatty acid increase with decreasing temperature in membranes as well as seed storage lipids (Neidleman, 1987; Thompson, 1993). Analyses of mutant strains of *Arabidopsis* have shown that polyunsaturated fatty acids are required for low-temperature survival (Hugly and Somerville, 1992; Lemieux *et al.*, 1990; Okuley *et al.*, 1994, Miquel *et al.*, 1993; Iba, 2002),

Trienoic fatty acids (TAs) such as hexadecatrienoic (C16:3) and α-linolenic (C18:3) acids, are the major polyunsaturated fatty acid species in membrane lipids. Increasing TAs in chloroplast membranes has been shown to enhance low temperature tolerance in plants during the early growth stage (Iba 2002). Overexpressing the *Arabidopsis* chloroplast ω-3 desaturase genes *FAD7* and *FAD8* in tobacco increased TAs in leaf tissue (Nishiuchi and Ida, 1998), which resulted in higher chilling tolerance in the transgenic tobacco seedlings, but not in mature plants (Kodama *et al.*, 1995; Nishiuchi and Ida, 1998). However, the overexpression of an ER localized ω-3 fatty acid desaturase *FAD3* gene in tobacco, which led to higher TAs accumulation in phospholipids, the main constituents of extrachloroplastic membranes, did not produce significant difference in the transgenic plants resistance to chilling and freezing (Hamada *et al.*, 1998). Apparently the mechanism of low-temperature adaptation in plants probably involves both transcriptional and /or post-translational regulation of the desaturases involved in polyunsaturated formation. Since many desaturases are encoded by multigene families, it is possible that some plant species may have both cold-inducible and non-cold-inducible forms of the same class of desaturase enzyme and/or gene (Murphy and Piffanelli, 1998).

Leaves of transgenic tobacco plants with additional desaturases genes showed significant increase in chilling tolerance compared to their wild-type counterparts (Kodama *et al.*, 1995; Ishizaki-Nishizawa *et al.*., 1996). Enzymatic activities of both ω-6 and ω-3 desaturases significantly increased in soybean (*Glycine max*) cell suspensions when the cultures were incubated at low temperatures (Cheesbrough 1989). *FAD2* was shown to be required for normal *Arabidopsis* growth at low-temperatures (Miquel *et al.*,1993), but no changes in *FAD2* transcript levels were observed when plants were transferred from 22 to 6 °C (Okuley *et al.*, 1994) A post-transcriptional control mechanism is likely for the temperature-dependent regulation of the *Arabidopsis FAD2*-encoded ω-6 desaturase.. *Arabidopsis* chloroplast ω-3 fatty acid desaturases, *FAD7* and *FAD8* genes, are significantly up-regulated by cold temperatures and salt stress. *FAD8* omega-3 desaturase gene expression changes in response to a change in ambient temperature, whereas the expression of *FAD7*, a second chloroplast omega-3 desaturase, is not affected by temperature. The expression of the *FAD8* gene is swhiched on and off by a difference of as little as a few degrees Celsius on either side of 25ºC (Gibson, *et al.*, 1994). This suggests that this temperature regulation operates via a mechanism that is quite different from that governing the expression of temperature-dependent genes, such as HSP genes, found so far. Other studies with Arabidopsis *FAD7/FAD8* double mutant indicated that is unlikely that *FAD8* gene expression is simply regulated at the transcriptional levels, as in the bacterial desaturase genes (Suzuki *et al.*, 2000).

In contrast, decreasing the amount of TAs in chloroplast membranes has been shown to strongly enhance high-temperature tolerance in plants. Co-suppression by gene silencing of the chloroplast ω-3 desaturases in transgenic tobacco resulted in very low levels of TAs and increased levels of DAs in the chloroplast membranes. When compared to control plants, these transgenic plants demonstrated resistance to high temperature (36ºC) that was not transient. Moreover, thermal desnaturation of photosynthetic machinery proteins was observed in the chloroplasts membranes of wild-type plants exposed to high temperatures but not in transgenic plants with decreased TAs (Iba, 2002).

The effect of elevated temperature on the fatty acid composition of storage lipids have been examined extensively in developing seeds. For example, the changes in soybean seed glycerolipid composition observed at high temperatures (Wilson 2004, Rajcan *et al.*, 2005; Hou *et al.*, 2006) were an increase in oleic acid (C18:1) and a decrease in polyunsaturated fatty acid (C18:2 + C18:3), a pattern similar to the one for plant leaves acclimating to rising temperatures (Upchurch 2008).

1.3.1.2. Salinity effect on cellular membrane stability and FAD activity

Kasunov (1998) reported that a reduction on total cell activity occurs during salt stress, being this reduction more evident in glycophytes. First the plant cell loses its principle mechanism of energy accumulation due to ATP splitting, and then a separation of oxidation and phosphorilation promotes the peroxidation of membrane phospholipids, originating free radicals. As a consequence, membrane structure deteorates and its permeability and selectivity changes (Kasunov, 1998; Zhu, 2003).

Ions can interact with the soil and the plant in different ways, which can lead to deficiency or toxicity phenomena that affect growth and development (Nilsen & Orcutt, 2000; Zhu, 2003). The ionic uptake by the cell is affected by the environmental salinity, which affects the relative availability of the ions in the area surrounding the root (Grattan & Grieve, 1999; Nilsen & Orcutt, 2000). With increasing salinity, the plants need more Calcium (Ca). Simultaneously, the transport of this ion can be reduced by ionic interactions, precipitation and the higher ionic strength of the medium, which leads to a reduction of the Ca availability for the plant (Grattan & Grieve, 1999; Nilsen & Orcutt, 2000). When Ca and Mg concentrations in the medium fall below the critical limits of the plant cell, the K uptake also decreases (Grattan and Grieve, 1999).

The hydrophobic lipid interior of the membrane acts as a barrier to the passage of many ions and large molecules. Moreover, membrane integrity and the functionality of integral membrane proteins (for example the photosynthetic machinery proteins) are maintained by membrane structure and fluidity. Non tolerant plants subjected to salt stress commonly show decrease levels of linolenic acid (18:3) in their membranes. Experiments with transgenic tobacco cells and plants demonstrated that overexpression of ω-3 desaturases, which increases linolenic acid, increases tolerance to salt and drought stress (Zang *et al.*, 2005; Im *et al.*, 2002; Upchurch 2008). This suggests that tolerance of plants to salt and drought is, to a large degree, dependent on the inherent level of fatty acid unsaturation and/or the ability to maintain or adjust fatty acid unsaturation (Berberich *et al.*, 1998; Mikami and Murata 2003). Further support for the importance of fatty acid unsaturation in salt stress is provided by transgenic research with *Saccharomyces cerevisiae*. The introduction of two sunflower (*Helianthus annuus*) ω-6 desaturases (*FAD2*-1, *FAD2*-3) into yeast, which can only synthesize monounsaturated, resulted in the production of dienoic fatty acids, an increase unsaturation index and fluidity, increased tolerance to NaCl and increased tolerance to freezing (Rodríguez-Vargas *et al.*. 2007).

1.3.1.3. Mechanical injury: wounding effect on cellular membranes and FAD activity

Plants have evolved both constitutive and inducible ways to resist stresses. Fatty acids are crucial components of cellular membranes, suberin, and cutin waxes that provide structural barriers to the environment (Beisson *et al.*, 2007). They contribute to inducible stress resistance through the remodeling of membrane fluidity (Iba, 2002), the release, though lipase activity, of α-linolenic acid (Grechkin, 1998), and as modulators of plant defense gene expression (Kachroo *et al.* 2001).

JA and methyl jasmonate participate in the signal regulation of a number of plant processes including wound and pathogen defense response. The release of linolenic acid from plant membrane lipids by stress activated lipases is through to provide the substrate for lipoxygenase and subsequent octadecanoid (oxylipin) pathway synthesis of jasmonic acid (JA) and methyl jasmonate (Padham *et al.* 2007). Efforts have been successful to identify and characterize fatty acid-deesterifing lipases that are activated by pathogen attack and/or environmental stress. Results suggest that both A1 and A2 phospholipases (Grechkin 1998, Padham *et al.*, 2007) are involved in linolenic (C18:3) mobilization from plant membrane lipids (Upchurch, 2008). Characterization of the DAD1 has shown it to encode a novel chloroplast A1 phospholipase that catalyses the release of linolenic acid from phospholipids (Ishiguro *et al.*, 2001). Both activities of A1 and A2 activities are induced in tomato leaves by wounding, treatment with the wound signal systemic, and treatment with oligosaccharides elicitors which suggests involvement in the lipid-mediated signaling response to herbivore and pathogen attack (Narvárez-Vásquez *et al.*, 1999). *FAD7* and *FAD8* are also induced by wounding and pathogen attack resulting in a marked accumulation of TAs and JA in tobacco plants (Nishiuhi and Iba, 1998).

1.4. Plant cell culture

Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavor and flagrance ingredients, food additives, and pesticides. The search for new plant-derivatives chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolisms (Rao and Ravishankar, 2002; Mulabagal and Tsay 2004).

Plant tissue culture techniques are essential to many types of academic inquiry, as well as to many applied aspects of plant science. Currently, tissue-cultured plants that have been genetically engineered provide insight into plant molecular biology and gene regulation. For example, selected plants can be cloned and cultured as suspended cells from which plant products can be harvest (Mineo, 1990).

Many plant species can be regenerated in vitro through several approaches but all require a starting point. This can be anything from a single cell that can be reproduced, a tissue or organ part, or a cut out piece of differentiated tissue (or organ) known as an explant. There are many different types of plant cultures; some of these culture types include the embryo culture, organ culture, *callus* culture, and cell culture. Figure 1 shows schematics of the different types of culture.

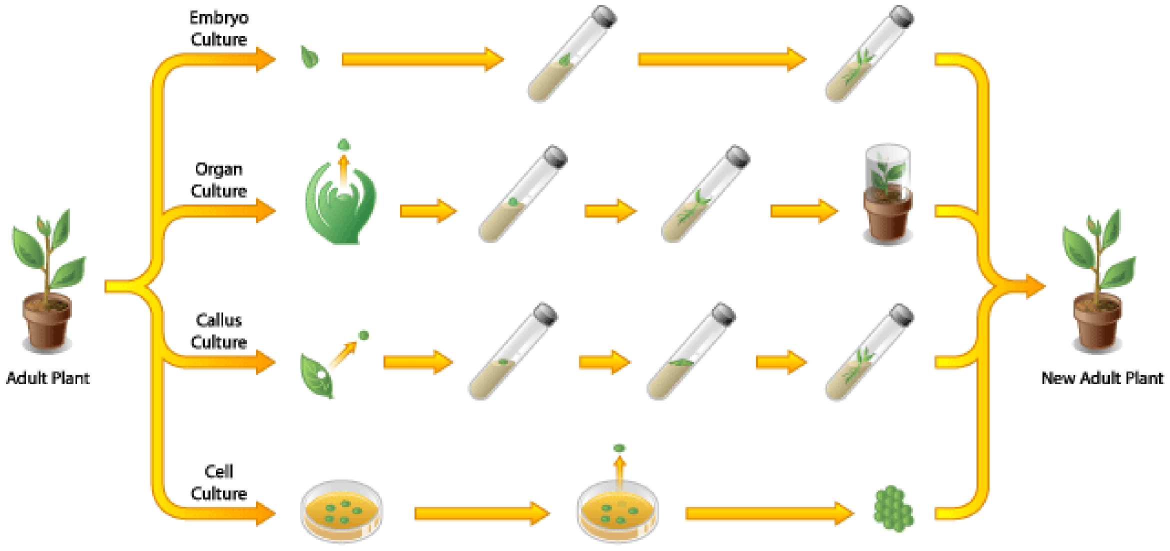


Figure 1.4.1. Different methods of cell culture. A) Embryo culture. Cells from the embryo are grown on agar media and allowed to develop into plants. B) Organ culture. Some specialized tissues are able to regenerate the whole plant. For example, the meristem tip can be excised from a plant and used to generate a whole new plant. C) *Callus* culture results when specific tissues are excised from the adult plant and allowed to form a structure known as a *callus*. The *callus* can then be used to generate a new tissue. D) Plant cell culture involves the dissociation of cells into a single cell suspension from where new plants can be generated. http://www.scq.ubc.ca/your-guide-to-plant-cell-culture).

The main culture of interest is the cell culture, which allows for the culture of isolated cells from a very small cluster dispersed in a liquid media. Cell suspension cultures are rapidly dividing homogenous suspensions of cells grown in liquid nutrient media from which samples can be taken (King, 1984). The *callus* are suspended in a liquid *callus* induction media containing all the required nutrients and elements to allow for optimal growth which acts to turn all cells into undifferentiated cells. Cells are grown on a media containing hormones to activate specific cell growth.

Hormones are produced by organisms and control such varied activities as growth, control of cell cycle, and reproduction. Many hormones have the ability to change the type of genes that are expressed with phenotypic consequences. Thus, they play an important role in cell differentiation.
Cytokinines genes are present in almost all higher plants as well as mosses, fungi, bacteria, and also in tRNA of many prokaryotes and eukaryotes. Cytokinines concentrations are highest in meristematic regions and areas of continuous growth potential such as roots, young leaves, developing fruits, and seeds, (Arteca, 1996; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992). Cytokinines stimulate cell division, morphogenesis (shoot initiation/bud formation) in tissue culture, stimulate the growth of lateral buds-release of apical dominance, leaf expansion resulting in cell enlargement, and promote the conversion of etioplast into chloroplast via stimulation of chlorophyll synthesis. The response will vary depending on the type of cytokinines and plant species (Davies, 1995, Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992).
Auxins are the first plant hormones discovered. Compounds are generally considered auxines if they can be characterized by their ability to induce cell elongation in stems and otherwise resemble indoacetic acid (first auxine isolated) in physiological activity. Auxines stimulate cell elongation, cell division in the cambium, root initiation on stem cuttings, and lateral root development in tissue culture. In combination with cytokinines, auxins stimulate differentiation of phloem and xylem, and *callus* development. By manipulating the proportions of these two hormones, *callus* tissue can be induced to produce shoots, roots or both, resulting in a regenerated plant. It has been found that if the cytokine-to-auxine ratio is maintained high, certain cells are produced in the *callus* that give rise to buds, stems and leaves. But if the cytokinines-to-auxine ratio is lowered, root formation is enhanced. By choosing the proper ratio, the *callus* may develop into a new plant. This control is a powerful tool to select plants for genetic engineering that demonstrate resistance to drought, salt stress, pathogens, harvesting techniques, and certain herbicides.

1.4.1. Examples of plant cell culture for production of bioactive compounds

Cell cultures are useful for the secondary metabolites they produce. Some of these metabolites that are a valuable source include flavors, natural sweeteners, industrial feedstock, perfumes and commercial insecticides. These products do not perform vital physiological functions like amino acids or nucleic acids, but they are produced to ward off potential predators, attract pollinators, or combat infectious diseases (Chawla, 2000). Although there are limitations of cell culture systems in producing secondary metabolites, they are favored over conventional cultivation methods. This is because of their ability to produce useful compounds under controlled conditions as well as their capability of using this technique to produce chemicals to meet market demands. In addition, specific cells of a plant can be multiplied to produce a higher yield of specific metabolites, which cannot be done through conventional methods of cultivation.

Discovery of cell cultures capable of producing specific medicinal compounds, at a rate similar or superior to that of intact plants have accelerated in the last few years (Mulabagal and Tsay, 2004). Some examples are saponins (Andrijani *et al.*, 1999, Desbene *et al.*, 1999), caffeine (Waller *et al.*, 1983), diterpenoids (Orihara *et al.*, 2002), triterpenes (Taniguchi *et al.*, 2002; Ayabe *et al.*, 1990) flavanoids (Ayabe *et al.*, 1990), alkaloids (Ikuta and Itokawa, 1988; Rech *et al.*, 1998, Sierra *et al.*, 1992), nicotine (Mantel *et al.*, 1983), morphine and codein (Siah and Doran, 1991), betacyanin (Sakuta *et al.*, 1987) among other, from different plant species.

Recent advances in the molecular biology, enzymology, and fermentation technology of plant cell culture suggest that these systems will become a viable source of important secondary metabolites.

1.4.2. Plant cell culture for production of polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are essential fatty acids in human nutrition due human's inability to perform desaturase-catalyzed formation of linoleic acid from oleic acid. Unsaturated fatty acids in organisms are elongated or further unsaturated to physiologically active substances such as prostaglandins or leukotrienes (Bajpai and Bajpai, 1992, 1993). Based on the potential need for PUFAs in human nutrition and medicine, searches for new sources of these compounds have been conducted. Plant seeds and nuts are rich as sources of fatty acids. However, because PUFA production in plants is strongly influenced by meteorological factors (Lajara *et al.*, 1990), plant cell cultures offer an attractive alternative for stable production of PUFAs. In vitro cultures of some PUFA-containing bryophytes have also been reported (Takio *et al.*, 1986; Hansen and Rossi, 1991). Plant cell culture such as *Ribes rubrum* was reported to produce 59.7% of its total lipids as PUFAs when cultivated at 10 ºC (Hirano *et al.*, 1997); Hartmann *et al.* (1986) reported that culture of moss (*Leptobryum pyriforme*) ptotonema contained up to 20% arachidonic and 7% eicosapentaenoic acids in its lipids while biomass yield was as low as 1.5 g l-1 for 20 days of culture. Considering that proliferation efficiency is a potential limiting factor in using plant cell culture for PUFA production, it is plausible to improve PUFA production by a better understanding on how PUFA biosynthesis is related to cell growth and by enhancing cell growth.

2. Scope of this Thesis

In spite of extensive studies made on the fatty acid concentration in this plant, no attempt was made, to my knowledge, to study at a molecular level the expression patterns of the desaturases and how the plant environmental growth conditions affect the expression and activities of these enzymes, essential in the biosynthesis of polyunsaturated fatty acids. This project aims to fill in the knowledge gap for this useful plant.

The hypothesis on which this research project is based are the follow:

Environmental salinity affects plant growth, nutrient uptake and accumulation in plant tissues.

Portulaca oleracea is an important vegetable source of polyunsaturated fatty acids

Desaturase gene expression partially governs leaf and seed fatty acid composition

Desaturase genes may differ in enzyme activity

Environmental conditions such temperature, salinity and wounding, regulates desaturase gene expression and fatty acid accumulation in leafs and seeds.

Based on these, the following objectives were set out:

(i) Determine the effect of salinity on plant yield and nutrient accumulation in *P. oleracea* leaves, by analyzing the plant yield, proximal nutritional analysis and ion accumulation in purslane leaves of plants exposed to chloride-salinity stress.

(ii) Study the fatty acid biosynthetic pathway in purslane, targeting fatty acid desaturases expression/activity in *P. oleracea* L. tissues. Develop gene-specific primers for FAD genes involved in the C18 fatty acid biosynthetic pathway in *P. oleracea* tissues.

(iii) Determine the effect of salinity on the expression of fatty acid desaturase genes by quantifying transcript accumulation and fatty acid accumulation in *P. oleracea* leaves.

(iv) Determine the effect of chilling and wounding stresses on the expression of fatty acid desaturase genes by quantifying transcript accumulation and fatty acid accumulation in *P. oleracea* tissues.

(v) Heterologous expression of *FAD* genes in *Saccharomyces cerevisiae* to estimate enzyme activity.

(vi) Develop cell culture lines of *P. oleracea* L. for in vitro production of polyunsaturated fatty acid and/or other active compounds present in this plant tissue

3. Effects of Salt stress on yield and nutritional composition of purslane (*Portulaca oleracea L.*).

Plants need essential mineral elements for their growth and development, but the presence of soluble salts in excess in the soil is harmful to the majority of plants. In fact the most limiting factor on plant growth, world wide, is soil salinity. (Zhu, 2003; Singh and Chatrath, 2001). The main factors responsible for soil salination are the low quality of irrigation waters, excessive fertilization and the deficient drainage of some soils. As a result the crops yield decreases and it is no longer possible to grow more salt-sensitive species (Singh and Chatrath, 2001; Grieve, 2000). An approach to the salinity problem is the use of crops with a higher tolerance to soil salinity (Shannon and Grieve, 1999; Neumann, 1997). The genus Portulaca, with over 100 species is found in every region of the world. Purslane (*Portulaca oleracea* L.) is a very versatile plant; it colonizes all kinds of soils (Kamil *et al.*, 2000; Matthews *et al.*, 1993), and has been rated as moderately tolerant (Kumamoto *et al.*, 1990; Maas and Grattan, 1977). The nutritional properties of purslane (Simopoulos, 2004; Palaniswany *et al.*, 2002; Simopoulos *et al.*, 1995) and its tolerance to saline environment make this plant a promising candidate for culture in soils affected by sea water intrusion whereas other cultures couldn't be established.

3.1. Salinity assay in soil culture: *P. oleracea* chemical composition analysis

The objective of this study was to determine the influence of saline stress on the chemical composition of purslane (*Portulaca oleracea*), in particular the mineral composition (**Paper I**). Purslane plants were cultured in a glasshouse, with natural light, at two different seasons spring (March) and summer (July). Air temperatures and humidity in the glasshouse were monitorized during both assays. In the spring assay, temperatures ranged from 5-25 °C (mean = 20 °C) in the spring test and 25-40 °C (mean = 30°C) in the summer experiment. Relative humidity ranged from 16-87 % in both seasons. During the first 6 weeks, the cultures were fed with a control nutritional solution design to improve fatty acid accumulation on purslane tissues. 6-week-old plants were then exposed to four salinity levels using irrigation solutions with electric conductivity values (ECi) of 0.8, 6.8, 12.8 and 24.2 dS.m-1. Samples of full grown leaf and stems of purslane were harvested after 7 and 15 days of the saline treatment exposure. Salinity affected the growth and yield of purslane plants, although a yield reduction was only observed in plants exposed to the highest level of salinity. Chemical analysis (DM basis) of leaves showed significant differences among the different saline treatments for all of the characteristics measured. Salinity levels, planting date and harvest time significantly influenced ($p<0.05$) levels of crude protein, total lipids, ash and carbohydrate content. Salinity treatments did not significantly ($p<0.05$) affect the water content of purslane leaves. The crude protein content of purslane leaves decreased with increasing salinity levels and time of exposure to treatment. On the other hand, carbohydrates and mineral residue content increased. An unusual phenomenon was noted for intermediate salinity levels whereby, an increase in total lipid content was measured in leaves of plant exposed to salinity treatments of 6.8 and 12.8 dS m^{-1}. This oil content increase is a positive outcome of salinity exposure, and it will be interesting to study, in the future, the detailed fatty acid profile of lipid fraction. The highest mineral residue content was seen in leaves of purslane exposed to the highest salinity treatment. We also observed a preferential accumulation of Ca and Zn in the leaves, while K and Na values were higher in the stems. When exposed to elevated salinity levels, potassium, calcium and zinc levels fell, and sodium, magnesium and chloride levels increased. This led to a significant increase in the Na/K, Mg/K, Na/Ca, and Mg/Ca ratios and was reflected in the reduction of growth and yield of purslane plants exposed to the more extreme salinity treatment. The lacks of yield reduction on the cultures exposed to salinity treatments may have to do with the short period of time in which the treatments were applied.

This study reveals that purslane is relatively tolerant to conditions of moderate salinity, being particularly well suited for cropping in areas where irrigation is necessarily carried out with water having an elevated chloride-based salinity. This ability improves its potential to become a key vegetable crop for animal and human consumption.

3.2. Salinity assay in hydroponics culture: oxalic acid and fatty acids accumulation in the leaves of two *P. oleracea* cultivars

During the first year of work a hydroponics system for purslane production was developed and it was implemented for salinity studies. In this study were used sodium chloride concentrations in range of 20-120 mM and physiological plant growth parameters were determined. Samples of those plants were stored and used in determination of total oxalic acid and fatty acids composition. Both varieties of "Golden leave" (GL) and "Green leaves" (GR) were used.

Twenty one days-old seedlings of purslane were transplanted into a closed hydroponics system in the greenhouse. Nitrogen at 200 μg ml^{-1} was provided as NO_3^- and NH_4^+ forms to yield NO_3^-—N: NH_4^+ ratio of 0.5:0.5. The nutritive solutions also contained macro nutrients (in μg ml^{-1}) 31 P, 207 K, 200 Ca, 48 Mg, and 64 S, and the micronutrients (in μM) 2 Na, 50 Cl, 24 B, 2 Mn, 2 Zn, 0.5 Cu, 0.5 Mo and 50 FeEDTA. Five saline treatments were applied: 20, 40, 60, 90, and 120 mM NaCl; the control had only the base nutritive solution. Eight plants of each cultivar were randomly placed in each tank, fulfilling a total 8 plants for each treatment replication. The nutrient solutions in the hydroponics system were aerated for 15 minutes every 2 hours using a time-controlled air bubbler. The solution pH was analyzed at 2 days intervals and maintained at 6.6-6.8 by adding 0.5M HCl or NaOH as needed. Two plants were randomly selected and harvest after 15 and 30 days of saline treatment. A sample of 50 g of fresh tissue (leaves) of *P. oleracea* leaves was dehydrated in an oven at 60 ºC for 48 h. The dehydrated tissue was macerated and stored in 15 ml vials at room temperature, protected from light until further analysis.

The plants were randomly grouped in a complete design with 4 replicas per sample. Two plants per treatment were used. Data was analyzed by SAS General Linear Models, version 8.1. To test the interaction of variety (V), exposed salinity levels (S) and harvest time (H), in the total oxalic acid (TOA) content of *P. oleracea* leaves, the ANOVA test was applied, considering 3 independent parameters (Variety, salinity levels, and harvest time). The differences between treatments were analyses by the Least Significant Difference (LSD) method, using t-student test. To test the interaction of variety (V) and exposed salinity levels (S) in the fatty acid methyl esters (FAMEs) content of *P. oleracea* leaves, the ANOVA test was applied, considering two independent parameters (Variety and salinity levels). The differences between treatments were analyzed by the Least Significant Difference (LSD) method, using t-student test. Values followed by different letter indexes denotes significant difference and those by the same letter index denote no significant difference at $P<0.05$. The interaction of the variety of the plant (V) and salinity levels (S) in the fatty acids content of *P. oleracea* leaves was statistically evaluated using SAS General Linear Models.

3.2.1. Oxalic acid analysis

Total oxalic acid (TOA) concentration was determined in the leaves of purslane plants submitted to 15 and 30 days of saline treatment. All determinations were conducted in four replications, by the procedures described by Savage et al (2000). One gram of finely grounded dehydrated plant material was weight into a 250 ml beakers and 50 ml of 2M HCL solution was added. The beakers were placed in a water bath at 80 ºC for 15 minutes. The extract was allowed to cool to room temperature and then transferred quantitatively to a 100 ml volumetric flask and made up to volume with 2 M chloride acid (HCl) solution. Three extractions were carried out for each sample. The extracts were then centrifuged at 3000 rpm and 10 ml of the supernatant was filtered throw 0.45 mm cellulose acetate membrane (Satorius, Goettingen, Germany). A 5 µl sample was analyzed using a Waters Chromatography System, consisting of a Waters 600 MS Isocratic/Gradient Pump and a Waters UV-VIS detector set at 210 nm. Data capture and processing were carried out using the Millenium Chromatographic Software. The chromatographic separation was carried out using an Aminex Ion Exclusion HPX-87H 300x7.8 mm analytical column attached to an Aninex Cation-H Gard column using an isocratic elution at 0.5 ml/min with 0.0125 mM Sulfuric acid as a mobile phase. The analytical column was held at room temperature ant the columns were equilibrated at a flow rate of 0.1 ml/min prior to use and in between sample sets. Before use the mobile phase was filtered through a 0.45 µm membrane and degassed using vacuum. The oxalic acid peak was identified by comparing the retention time to a range of commune plant organic acids standards. One standard curve was prepared in the range of 1-20 mg/100 ml and it was used for the determination of TOA in the samples. All blank and standard solutions were filtered throw a 0.45 mm cellulose acetate membrane syringe filter prior to analysis.

Total oxalic acid (TOA) concentrations (means) from all samples are summarized in Table 3.2.1.1.

Table 3.2.1.1. Total oxalic acid concentration in *P. oleracea* leaves exposed to 15 and 30 days of saline stress

[NaCl] mM	oxalic acid mg/g DW [1]			
	Golden Leaf		Green Leaf	
	15 days	30 days	15 days	30 days
0	79.9 ± 4,9	113.8 ± 5.0	63.6 ± 4.0	111.9 ± 4.3
20	66.3 ± 1,5	79.6 ± 2.8	65.6 ± 6.0	117.9 ± 7.1
40	49.8 ± 2.6	64.6 ± 6.3	60.6 ± 4.4	92.8 ± 3.1
60	36.6 ± 2.8	62.3 ± 4.1	44.7 ± 2.6	73.6 ± 8.7
90	29.1 ± 4.1	55.2 ± 4.4	37.1 ± 2.7	43.8 ± 1.7
120	26.7 ± 4.3	32.7 ± 4,.6	21.4 ± 0.5	41.5 ± 2.6

[1] The values corresponded to the mean value of four determinations, affected by the standard variation value.

For the control plants the TOA content of *P. oleracea* leaves at 15 days was slightly higher (0.85 mmol OA/g DW, GL variety; 0.71 mmol OA/g DW, GR Variety) that the reference value reported by Palaniswamy *et al.* (2002), 0.7 mmols/g DW, when similar levels of NO_3^-—N: NH_4^+-N were used as nitrogen(N) source in the nutritive solution In the other hand, at 30 days of treatment, TOA content was higher (1.22 mmol OA/g DW, GL variety; 1.24 mmol OA/g DW, GL variety), similar to those obtain when the only source of nitrogen in the medium was the nitrate form (NO_3^-—N), 1.0 mmols OA/g DW (Palaniswamy *et al.*, 2002). When nitrogen (N) is provided in a nitrate form, the nitrate has to be reduced in the shoots (nitrate reduction by nitrogen redutase) before the N can be used by the plant. This reaction results in the production and accumulation of organic acids such as oxalic acid (Libert and Franceschi, 1987) in the leaves and stems. Since there was no replacement of nutritive solution during this assay, it is possible that the plant consumed all the nitrogen (N) in the NH_4^+-N first and then started to use the NO_3^-—N form, what can explain the increase of TOA in the plants leaves in the latest harvest. In future studies the nutritive solution in the hydroponics system should be total or partial replaced in weekly base, to prevent nutrient exhaustion.

The interaction of the studied parameters: variety of the plant (V), sodium chloride concentration in the hydroponics nutritive solution (S), and the time of harvest (H) in the total oxalic acid (TOA) content in *P. oleracea* leaves, was statistically evaluated using SAS General Linear Models. The statistical analysis shows that all parameters significantly affected ($p<0.0001$) the total oxalic acid concentration, with a correlation level of 98.5% (R^2= 0.985).The combined effects of variety and salinity level, as well as salinity level and harvest time, were also significant ($p<0.0001$).

The t-Student test was applied to the data, using the LSD methodology. This analysis shows that the mean TOA content in leaves of plants harvest after 30 days of saline stress exposure were significantly higher ($p<0.05$; LSD = 2.3573) then the TOA contents of leaves from plants harvest 15 days after saline stress exposure. The variety of *P. oleracea* also significantly ($p<0.05$) influenced the TOA contents, the leaves of plants from the "Green Leaf" (GR) variety had higher levels of TOA compared to plants of the "Golden Leaf" (GL) variety. It was observed a significant ($p<0.05$) decrease of TOA content in leaves of *P. oleracea* with the increase of sodium chloride (NaCl) concentration of the nutritional solution in the hydroponics system (0 mM^a > 20 mM^b >40 mM^c >60 mM^d >90 mM^e >120 mM^f). Purslane accumulates chloride ions in the leaves when exposed to saline environment (Teixeira and Carvalho, 2008), so it is possible that the decrease of oxalic acid accumulation on the leaves can be associated to a competitive accumulation between oxalic acid and chloride ion.

3.2.2. Fatty acid Analysis

Fatty acid composition was determined in leaves of purslane plants exposed to saline treatments for 30 days according with the procedures described by Liu, *et al.* (2000). A 1-g amount of dehydrated plant tissue was mixed with 6 ml of chloroform-methanol (2:1 v/v) containing internal standard (I.S.; C21:0; Sigma, USA; 0.2 mg/ml) and 1 % butylhidroxytoluene (BHT) to prevent oxidation of fatty acids. The mixture was vortex-mixed for 5 minutes and centrifuged at 3000 g for 10 minutes at 5 ºC. The lower phase (1 ml) was collected and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 2 ml of methanol-hexane (4:1, v/v) and acetyl chloride (0.2 ml) was slowly added while vigorously stirring. Fatty acids were esterified at 100 ºC for 1 h, then cooled in water and the reaction stopped by addition of 6% K_2CO_3 solution (5 ml). The samples were vortex-mixed, centrifuged and the upper phase was collected prior to GC analysis. Samples (1 µl) were injected via an auto sampler onto a fused-silica capillary column (Supelco; Omegawax 250; 30 m x 0.25 mm I.D., 30 µm film thickness) in a HP 6890 gas chromatograph (GC-MS) system fitted with a flame ionization detector and eluted with helium (He) at 44.0 ± 1 ml/min, with a split ratio of 1:17. The injector and detector were heated to 250 ºC. The column was temperature programmed from 130 ºC (hold 1 min) to 180 ºC at 25 ºC/min, and then to 230 ºC (old 7 min) at 2.5 ºC/min. Fatty acid methyl esters were identified by comparing GC retention times with those of a mixture of standard fatty acids methyl esters (FAMEs) Mix C14-C22, (Supelco). Fatty acids were quantified using peak areas integration against internal standard.

Fatty acid composition was determined in leaves of purslane plants exposed to saline treatments for 30 days. The fatty acids identified in the samples of *P. oleracea* leaves were palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1), linoleic acid (LA, C18:2), α-linolenic acid (ALA, C18:3), araquidonic acid (ARA, C20:0), and behemic acid (BEH, C22:0). The results obtain in this analysis are summarized in Table 3.2.2.1.

Table 3.2.2.1. Fatty acid contents (mg/g dry mass) of *P. oleracea* leaves of plants submitted to 30 days of saline treatment exposure. *C16:0* -palmitic acid *(*PA), C18:0-stearic acid (SA), C18:1-oleic acid (OA), C18:2-linoleic acid (LA), C18:3-α-linolenic acid (ALA), C20:0-araquidonic acid (ARA), and C22:0-behemic acid (BEH).

		Fatty acids contents (mg /g DW[1])							
[NaCl] mM	Sample	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	TOTAL
0	GL	19.1 + 3.5	1.3 + 0.1	1.5 + 0.2	5.1+ 0.6	16.1 + 1.4	0.5 + 0.04	0.4 + 0.07	45.6 + 2.7
	GR	27.4 + 0.1	1.6 + 0.3	1.6 + 0.3	5.3 + 0.3	16.2 + 0.8	0.5 + 0.15	0.4 + 0.11	54.5 + 0.3
20	GL	21.9 + 5.1	1.2 + 0.1	1.6 + 0,0	5.5 + 0.3	16.7 + 0.8	0.5 + 0.10	0.7+ 0.05	49.5 + 4.1
	GR	26.5 + 1.5	1.2 + 0.1	1.2 + 0,1	4.7 + 0.5	16.4 + 0.5	0.5 + 0.01	0.4 + 0.16	52.5 + 2.0
40	GL	24.4 + 2.5	1.2 + 0.1	1.5+ 0.1	6.1 + 0.5	17.0 + 0.7	0.5 + 0.04	0.7 + 0.03	53.0 + 3.1
	GR	26.9 + 0.6	1.2 + 0.1	1.2 + 0.1	4.7 + 0.4	16.4 + 0.8	0.5 + 0.07	0.5 + 0.07	52.7 + 1.6
60	GL	24.4 + 0.9	1.0 + 0.1	1.4 + 0.1	4.9 + 0.3	15.2 + 0.8	0.6 + 0.10	0.8 + 0.08	49.7 + 1.3
	GR	24.2 + 0.2	1.1 + 0.0	1.1 + 0.1	4.6 + 0.3	16.1 + 1.5	0.5 + 0.06	0.8 + 0.04	49.5 + 1.6
90	GL	24.6 + 3.2	1.0 + 0.3	1.0 + 0.3	4.7 + 0.2	14.5 + 0.7	0.7 + 0.06	0.8 + 0.07	48.1 + 2.3
	GR	23.9 + 0.8	1.1 + 0.2	1.1 + 0.1	4.5 + 0.3	14.5 + 0.2	0.8 + 0.04	0.8 + 0.12	48.0 + 2.7
120	GL	24.8 + 1.8	0.9 + 0.1	1.0 + 0.3	4.4 + 0.7	11.9 + 0.1	0.7 + 0.03	0.9 + 0.03	45.6 + 2.1
	GR	17.5 + 1.0	1.1 + 0.0	1.0 + 0.1	4.0 + 0.1	13.1 + 2.2	0.9 + 0.05	1.0 + 0.12	39.7 + 2.7

[1] Data are means ± S.E. (n=4).

In general, the total amount of fatty acids (FA) increases slightly with the increase of NaCl concentration until 40 mM NaCl, and decreases for higher concentration (Table 3.2.2.1). In the "Golden leaf" variety the highest concentration was obtained in leaves of plants submitted to 40 mM NaCl, 53.0 mg FA/g DW, decreasing for higher concentrations, although the fatty acid concentration of plants submitted to 120 mM treatment is identical to the concentration registered for the control plants, 45.6 mg FA/g DW (0 mM NaCl). For the variety "Green leaf", the highest concentration of fatty acid was observed in the control plants, 54.5 mg FA/g DW, decreasing slightly with the increase of NaCl concentration of the hydroponics solution.

Palmitic acid was the major constituent in the lipid mixture, and its proportion increased with higher NaCl treatments from 42 % in control plants to 54.4 % TFA in plants submitted to 120 mM NaCl. The second major fatty acid present is the α-linolenic acid (ALA, C18:3), with the highest concentration in control plants, 35.4 % and decreases with the increase of NaCl concentration of hydroponics system solution. Linoleic acid (LA, C18:2) counts for 11 % for NaCl concentrations equal to or lower than 60 mM NaCl, decreasing slightly at higher concentrations of NaCl. These decreases of LA and ALA proportions are probably associated to an increase of membrane oxidation due the exposure of the cells to the saline environment. Low temperatures and salinity may stimulate the production of oxygen reactive species by altering the metabolism of electron transference, causing damages to the cellular membranes and the accumulation of tiobarbituric acid derivates (TBARs), associated to the membrane lipid peroxidation (Massardo *et al.*, 2000; Hodges *et al.*, 1999). No significant variation in oleic acid (OA, C18:1) content was detected, although a slight increase in its proportion, from 2.5- to 3.2 % was present (Table 3.2.2.1). There is also an increase of the long chain saturated fatty acids, arachidic acid (ARA, C20:0), from 0.8 % (control) to 2.2 % (120 mM NaCl) and behenic acid (BEH, C22:0), from 0.7 % (control) to 2.4 % (120 mM) (Table 3.2.2.1).

The statistical analysis showed that the salinity levels and the combined effect of salinity with variety, significantly affected the fatty acids content ($p<0.005$). On the other hand, by it self, variety of the plant did not significantly ($p<0.005$) affected the fatty acids content of *P. oleracea* leaves, with the exception of LA, which showed higher levels in the leaves of GL variety. The correlation level between the fatty acids content in leaves of *P. oleracea* and the tested parameter was relatively high, 72.4% (R2= 0.724). The t-Student test was applied to the data, using the LSD methodology. The NaCl concentration significantly affected the fatty acids contents in *P. oleracea* leaves with a high variation between the fatty acids proportion on the lipid mix. Palmitic (PA) and stearic (SA) acids did not show a particular variation pattern in response to salinity treatments. In the other hand, the oleic acid (OA) content in the leaves decreases significantly ($p<0.05$) with the increase of NaCl concentration for NaCl treatments equal to or lower than 60 mM NaCl (0 $\text{mM}^{a} \cong 20\ \text{mM}^{a} \cong 40\ \text{mM}^{a} \cong 60\ \text{mM}^{a} > 90\ \text{mM}^{ab} > 120\ \text{mM}^{b}$). The linoleic acid (LA) content was higher in the plants exposed to 60 mM NaCl and decreased for higher saline treatments ($60\ mM^{a} > 0\ mM^{ab} > 20\ mM^{abc} > 40\ mM^{bc} > 90mM \cong 120\ mM^{c}$). The α-linolenic acid (ALA) content did not significantly ($p<0.05$) differ from control plants for level equal to or lower than 60 mM NaCl, and decreased with the increasing salinity levels ($0\ mM^{a} \cong 20\ mM^{a} \cong 40\ mM^{a} > 60\ mM^{ab} > 90\ mM^{bc} > 120\ mM^{c}$). The araquidonic acid (ARA) content in the leaves significantly ($p<0.05$) increased with the increase of NaCl concentration in the hydroponics nutritive solution, showing highest content in the in plants exposed to 90 mM NaCl. There were no significant differences in the ARA and BEH for treatments equal to or lower than 60 mM NaCl with a significant increase ($p<0.05$) for higher NaCl concentrations.

3.2.3. Conclusions

In the first assay, conducted in soil culture system, we observed the salinity levels above the plant tolerance limit (60 mM NaCl) lead to a reduction in the culture yield and nutritional value, with a significant decrease in protein and lipid contents. We also detect a shift in ionic balance in stressed cultures: Na and Cl levels increased and Ca and K levels decrease in *P. oleracea* shoots in response to imposed salinity.

In the second assay, conducted in hydroponics culture system, we observed that the oxalic acid synthesis was negatively affected by the imposed salinity with lower level of accumulation in plants exposed to $NaCl \geq 90$ mM. The "Golden leaf" cultivar accumulated lower concentrations of total oxalic. Regarding fatty acid accumulation, different responses to the saline treatments were observed. The "Golden leaf" variety showed a decrease in the amount of unsaturated fatty acids, and an increased amount of the long chain saturated fatty acids, arachidic acid (C20:0) and behemic acid (C22:2), with the increase of NaCl concentration. In the "Green leaf" variety the pattern was not clear, and in the case of linolenic acid (C18:3) the inverse behavior was observed; with an increased amount of the linolenic content at higher concentrations of NaCl. The fraction of unsaturated fatty acids (PUFAs) in the lipid mixture was lower than expected, in control plants, although the proportions between OA, LA and ALA were maintained. These results suggest a possible degradation of the sample during the drying process, with some oxidation of the PUFAs. In future studies fresh or lyophilized plant material will be used for these determinations.

The overall data suggest that the "Golden leaf" variety is more suitable to study the effects of environmental stresses in the nutritional value of purslane, not only because it apparently accumulates lesser amounts of total oxalic acid in the leaves, but also because it showed a faster and more prolific growth in the hydroponics system.

In both studies, for NaCl treatments below 90 mM an increase in the total fatty acid content and polyunsaturated fatty acids was detected. This data reflects this plant tolerance to saline environments and ads to its potential use as a crop culture.

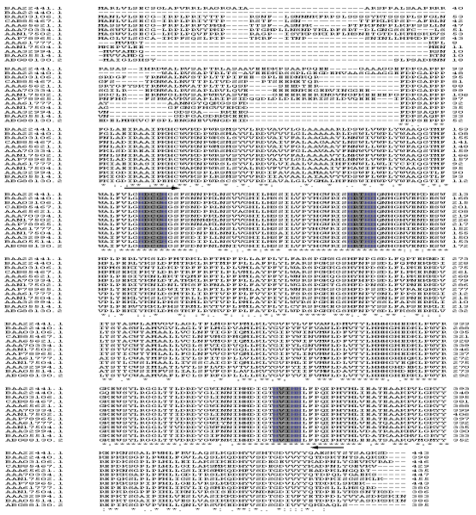
4. Fatty acid desaturase genes: molecular cloning, sequence analysis and gene expression patterns in *P. oleracea* (L.) tissues.

Purslane has a long history of use for human food, animal feed and medicinal proposes. Recent research indicates that purslane offers better nourishment than the major cultivated vegetables (Kumamoto *et al.*, 1990; Omara-Alwala *et al.*, 1991; Simopoulos, 2004; Palaniswany *et al.*, 2001). It is a rich source of omega-3 fatty acids, which is important in preventing heart attacks and strengthening the immune system (Simopoulos, 2004). In spite of extensive studies made on the fatty acid concentration in this plant, no attempt to study at a molecular level on expression patterns of the desaturases and how the plant environment affects the expression and activities of these enzymes, essential in the biosynthesis of polyunsaturated fatty acids. This project aims to fill in the knowledge gap for this useful plant.

4.1. Cloning protocol for fatty acid desaturases

In order to clone and characterize full length fatty acid desaturases from purslane, a PCR based cloning protocol was developed (Fig. 4.1.1) (**Papers II and III**). In the first step, an alignment of plant fatty acid desaturase sequences was made for each gene of interest. In Figure 4.1.1.-A is an example of the alignment for *FAD2* plant genes. This revealed three highly conserved regions, which were back translated and used as template to design a set of degenerated PCR primers for each target gene. These primers were then used in a PCR protocol with reversely transcribed purslane leaf mRNA as template (Fig. 4.1.1.-B). This first PCR gave 600 to 900 bp fragments, which showed homology to the respective plant fatty acid desaturases. To obtain the full length clone, a set of primers specific to the previous amplified fragment, were used in 5'- and 3' Rapid Amplification of cDNA Ends PCR (RACE PCR) with leaf total mRNA as template (Fig. 4.1.1.-C).

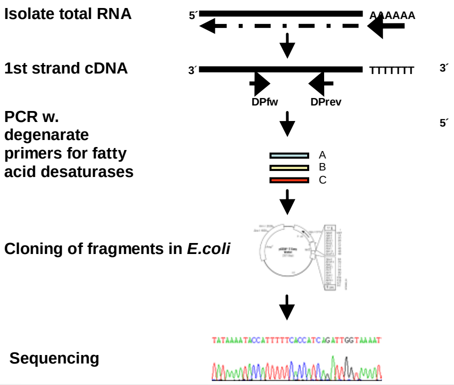
A- Alignment



Alignment of the coding sequence of omega-6 (A) and omega-3 (B) fatty acid desaturases

Two degenerated primers (*FAD2*fw1 and *FAD6* fw1) were designed and used together with the universal primer AUAP;, for amplifying a 800-900 bp fragment of two omega -6 FAD genes. A similar approach was conducted for the amplification of omega-3 FAD genes, One degenerated primers (*FAD3* Fw1) was design and used together with the universal primer AUAP, for amplifying a 800 bp fragment of three omega-3 FAD genes. For *FAD2*, *FAD3* and *FAD8* genes, further reverse degenerated primers were design

B- PCR with degenerate primers

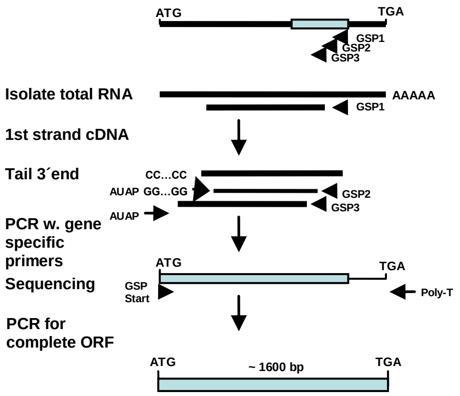


Total RNA from the plant is isolated and a 1 st strand cDNA synthesis is performed.

PCR with the degenerate primers is performed to amplify the internal 800-900 bp fragment. It is possible to amplify fragments from several different fatty acid desaturases at this step.

The amplified fragments are cloned and sequenced. Sequence analysis reveal how many different desaturases have been "fished" and heir homology to previously characterized fatty acid desaturases.

C- PCR with gene specific primers



From the sequence analysis a set of three gene specific primers is designed (GSP1-3).

First strand cDNA synthesis from total RNA is performed with GSP1.

The 3´end of the 1st strand cDNA is tailed with an adaptor sequence, to which a specific primer (AUAP) is matching. PCR is performed with GSP2 and 3.

After sequencing of the ATG region of the ORF a specific primer for this region is designed (GSP start) and used toghether with a poly-T primer to amplify the complete coding sequence (1200-1500 bp) from total RNA.

Fig.4.1.1. Cloning protocol for fatty acid desaturases. (A) Alignment and design of degenerated primers. (B) Isolation of internal section of different fatty acid desaturases. (C) 5'- and 3' RACE PCRs followed by amplification of the full length ORF.

Using these protocols we were able to amplify three full-length cDNA clones of plastid fatty acid desaturases, *PoleFAD6* (ω6-fatty acid desaturases), *PoleFAD7* and *PoleFAD8* (ω3-fatty acid desaturases), and two microsomal fatty acid desaturases, *PoleFAD2*-1 and *PoleFAD2*-2 (ω6-fatty acid desaturase). A partial cDNA clone of the microsomal oleate desaturase *FAD3* gene was also isolated, with a high degree of identity to other known plant *FAD3* and *FAD7* genes, here by designated by *PoleFAD3*-A In all translated peptide sequences the three histidine boxes domains typical of all membrane-bound desaturases where present in the correct positioning. In table 4.1.1 the main characteristics of the full cDNA sequences of all isolated genes.

Nucleotide sequences from cDNA clones and deduced amino acid sequences were identified by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Predictions of open reading frames (ORFs) were made using the DNALC Bioinformatics software (http://www.dnalc.org/bioinformatics/2003/2003_dnalc_nucleotide_analyzer.htm). Theoretical molecular weights of deduced polypeptides and hydropathy analysis were analyzed using the Peptide properties calculator software (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp)

Transmembrane regions were predicted by the TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Predictions of sub cellular localization of the deduced polypeptides were conducted by using PSORT (http://www.psort.nibb.ac.jp./form.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) algorithms.

Amino acid multiple alignments were made with the ClustalW program under default parameters. A phylogenetic tree was constructed using the neighbor-joining algorithm included in the ClustalW program. The tree was visualized using the Tree View program.

Table 4.1.1. Description of three full-length cDNAs encoding fatty acid desaturases from *Portulaca oleracea* L.

Name	Full-length cDNA (bp)	ORF (bp)	Encoded amino acid	Protein molecular mass (KDa)	Isoelectric point	Predicted sub cellular location
PoleFAD2-2	1232	1151	383	44.0	9.2	ER
PoleFAD2-1	1223	1148	381	43.8	8.7	ER
PoleFAD6	1573	1374	457	52.9	9.2	Chloroplast
PoleFAD7	1523	1338	446	50.5	7.5	Chloroplast
PoleFAD8	1606	1362	453	52.4	8.2	Chloroplast

Blast searches showed high similarities of the predicted *Portulaca FAD* genes amino acid sequences to several orthologues desaturases available in the GenBank database. *PoleFAD2*-2 and *PoleFAD2*-1 revealed 63-72% identity (68-83 % similarity) and 67-82 % (73-82 % similarity) to other *FAD2*, respectively. The sequence identity or similarity between *PoleFAD2*-l and *PoleFAD2*-s was higher, 87% identity compared to other orthologues sequences. *PoleFAD6* showed 64-70% identity (82-85% similarity) to *FAD6* proteins from different plant species. A low sequence identity between the two isoforms of *FAD2* gene and *PoleFAD6* was detected (17 or 19 %, respectively), indicating the high phylogenetic divergence of these three ω-6 desaturases.

The two isolated cDNA clones of omega-3 FAD, *PoleFAD7* and *PoleFAD8* amino acid sequences showed high similarity to other orthologous desaturases sequences available in GenBank database. *PoleFAD7* and *PoleFAD8* exhibited 68-76 % and 60-67 % of identity to other *FAD7* and *FAD8* proteins from different plant species, respectively. The sequence identity between *PoleFAD7* and *PoleFAD8* was 63 %. We were also able to isolate a partial cDNA clone that shown high similarity to the *PoleFAD7* (70%) and *PoleFAD8* (81%), and other omega-3 genes, (81-85% similarity), hereby designated as *PoleFAD3*-A.

4.2. Polypeptide Sequence analysis

In the present study, using a PCR-based approach, we have cloned five distinct and complete fatty acid desaturase cDNAs from purslane, designated as Po*FAD2*-l, Po*FAD2*-2, *PoleFAD6*, *PoleFAD7* and *PoleFAD8*; and one partial cDNA clone designated by *PoleFAD3*-A. *PoleFAD6* gene was highly divergent in the sequence structure, compared to the two isoforms of *FAD2* gene, and either of them resembles more closely ω-6 desaturase genes from different species than each other (Fig. 2 **PaperII**). Phylogenetic analysis revealed that the *FAD6* gene family, including *PoleFAD6*, forms a tight cluster of more related sequences to each other than to the members of *FAD2* subgroups (Fig. 2 **PaperII**). Both isoforms of *PoleFAD2* were highly similar to other ER localized ω-6 fatty acid desaturases, whereas *PoleFAD6* was highly homologous to other plant ω-6 fatty acid desaturases that have putative plastid transit peptides (Fig. 2 **PaperII**). These observations support the hypothesis that the evolutionary process of *FAD2* might be different from that of *FAD6* genes (Seffens *et al.*, 1990; Jin *et al.*, 2001). In addition, the relative high level of divergence within the *FAD2* group observed implies that certain genes may have evolved differently, although to further confirm details in phylogenetic relationships within this gene family more data are required.

Based on multiple alignments of their deduced amino acid sequences and phylogenetic analysis it was shown that both genes, *PoleFAD7* and *PoleFAD8* had a relatively high homology to other plant ω-3 fatty acid desaturases that have putative plastid transit peptides(Fig. 2 **PaperIII**).. The *FAD7* and *FAD8* genes of *Portulaca oleracea* L. have been isolated and characterized by genetic criteria as encoding chloroplast-localized omega-3 desaturases.

Besides the high homology to other published sequences found, eight histidines in three separate clusters were also detected in all isolated genes with the following consensus sequence: $HX_{(3-4)}HX_{(20-50)}HX_{(2-3)}HHX_{(100-200)}HX_{(2-3)}HH$. These conservative motifs were found in all membrane bound desaturase and contribute to an iron-binding site (Okuley et al,. 1994; Los and Murata, 1998; Murphy and Piffanelli, 1998). The predictions of three to four putative trans-membrane regions for FAD polypeptides also support the notion that all desaturases are membrane bound. Further sequence analysis showed that both isoforms of *FAD2* amino acid sequence has an aromatic amino acid-enriched sequence at the C-terminus (-YGNKS/ -YNNTL – Fig. 1A, **Paper II**) that has been found to act as an ER retrieval signal (McCartney *et al.*, 2004). As opposed, *PoleFAD6*, *PoleFAD7* and *PoleFAD8* possessed a putative N-terminus transit peptide of 65, 51 and 74 amino acids, respectively, capable of transportation the molecule into the plastids (Fig. 1B, **Paper II**; and Fig.1, **Paper III**). This N-terminal sequence also had several characteristics of plastid transit peptides, including a high content of hydroxylated residues (Ser, Thr and Tyr), a low content of acidic residues and the conserved N-terminal Met-Ala dipeptide (Liu *et al.*, 1999a,b). No transit peptide signal was identified in the N-terminus of *PoleFAD3*-A fragment, which may indicate that this fragment corresponds to an ER microsomal gene. It will be necessary to isolate the full-length clone to corroborate this fact. Three putative transmembrane domains were identified in the polypeptide sequence, which strongly suggests that this fragment corresponds to a membrane-bound protein.

4.3. Spatial expression of FAD genes in *P. oleracea* tissues

Real-time PCR is a variant of the classical PCR technique in which the amplified product is measured at each cycle throughout the PCR reaction. Real-time PCR may be used in quantification and detection of foreign DNA (e.g. transgenes or contaminating micro-organisms), and in gene expression studies (Gachon *et al.*, 2004). To determine the steady-state level *Portulaca* fatty acid desaturase genes expression in different tissues of purslane, RT-PCR analysis was carried out using total RNA extracted from young expanding leaves, mature leaves, young stems (tips), stems, root, root tips and seeds (Table.4.3.1), as a template (**Papers II and III**).

The synthesized first strand cDNA from different plant organs were used as templates for PCR to amplify a partial region of *PoleFAD2*, *PoleFAD3*, *PoleFAD6*, *PoleFAD7* and *PoleFAD8* genes, using a pair of sequence-specific primers (Table 4.3.1), design based on conserved regions of each target gene . Three housekeeping genes were tested to be used in semi-quantitative analysis, based on an equally expression response in all plant organs. Primer sets for *EF1* (elongation factor 1), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *UBI* (ubiquitin) housekeeping genes were used (Table 4.3.1).GAPDH was selected as a control gene for this assay because it showed constant transcript levels in all purslane tissues.

Table 4.3.1. Sequence specific primers Sets used in RT-PCR assay and expected size of the amplified DNA fragment

Gene	Primer designation	Primer sequence	Size of amplified fragment
PoleFAD2-1	*FAD2* RT-PCR fw	5’-GGGTTCGTCCAGGGCTGTGTGCT-3’	194 bp
	FAD2 RT-PCR rv	5’-CCACTGGAGGCCGGACTTTCGTTT-3’	
PoleFAD2-2	*FAD2* RT-PCR fw	5’-GGCTACATCCAAGGTTGCATCTT-3’	181 bp
	FAD2 RT-PCR rv	5’-CCACTTCATGCCATCCTTCTTCTT-3’	
PoleFAD3-A	*FAD3* RT-PCR fw	5`-CCTACCCTTGACCCAGAACAGGGTT-3`	174 bp
	FAD3 RT-PCR rv	5`-GATGAAAACACAGACAATGCAGC-3`	
PoleFAD6	*FAD6* RT-PCR fw	5’- GCC GTG CCA GAA GGG TTT CGG TA-3’	197 bp
	FAD6 RT-PCR rv	5’- CAC CTT AGG AAG GGA ACT GAT -3’	
PoleFAD7	*FAD7* RT-PCR fw	5`-GAGGTTCCCCTGTTTCCCCGGAT-3`	175 bp
	FAD7 RT-PCR rv	5`-AATGCGGTGGTCAATGGGTCGCC-3`	
PoleFAD8	*FAD8* RT-PCR fw	5`-CTCCCATCTTCTCTCATTTTCATT-3`	193 bp
	FAD8 RT-PCR rv	5`-TTAGGGTTTGATCATGGGGCACC-3`	
EF1[a, b]	EF1 RT-PCR fw	5`-CTGTAACAAGATGGATGCCACTAC-3`	200 bp
	EF1 RT-PCR rv	5`-CAGTCAAGGTTRGTGGACCT-3`	
GAPDH[a,c]	GAPDH RT-PCR fw	5´-CACGGCCACTGGAAGCA-3`	350 bp
	GAPDH RT-PCR rv	5`-TCCTCAGGGTTCCTGATGCC-3`	
UBI[a]	UBI RT-PCR fw	5`-GGCATGCAGATCTTTGTGAAGAC-3`	180 bp
	UBI RT-PCR fw	5`-GGGATACCCTCCTTGTCCTG-3`	

a – Housekeeping genes; b-Primer set referred by Bilyeu *et al.* (2003); c-Primer set referred by Iskandar *et al.* (2004)

Amplification was carried out using standard reaction conditions with SYBR Green mix (Applied Biosystems, USA) in a thermal cycler (7500 Real-Time PCR System (Applied Biosystems, USA)), programmed for 94 ºC/2 min and then 40 cycles of 94 ºC/30s, 55 ºC/30s, 72 ºC/1 min. The RT-PCR products were separated by 1% agarose gel electrophoresis. The RT-PCR fragments were cloned in E. coli and selected by PCR colony check, using the vector primers. Purified plasmids of putative positive clones of RT-PCR products were sent to sequence to confirm the amplified fragments sequence to Eurofins MWG GmbH (Germany).

Both ER and plastid FAD were expressed in all tissues of purslane studied (Fig.4.3.2), reflecting the crucial roles of these enzymes in fatty acid desaturation. The expression of the plastidial *PoleFAD6* and *PoleFAD7* genes was not restricted to photosynthetic tissues, as it was shown by its expression in root and seed tissues. *PoleFAD2*-2 and *PoleFAD3* genes shared, qualitatively, almost similar pattern of expression, with the highest transcript levels observed in actively developing young organs, i.e. shoot and root tips most likely to furnish the demand of polyunsaturated fatty acids in membrane biosynthesis.

All genes, with the exception of *PoleFAD8*, were detected in purslane seed, with higher transcription levels detected for *PoleFAD2*-2 and *PoleFAD6* (Fig. 4.3.1). Tao *et al.* (2006) also reported a high level of expression of *FAD2* gene in immature embryos (seeds) and lower level of expression of in photosynthetic tissues (leaves, stems and root) in maize (Zea maize).

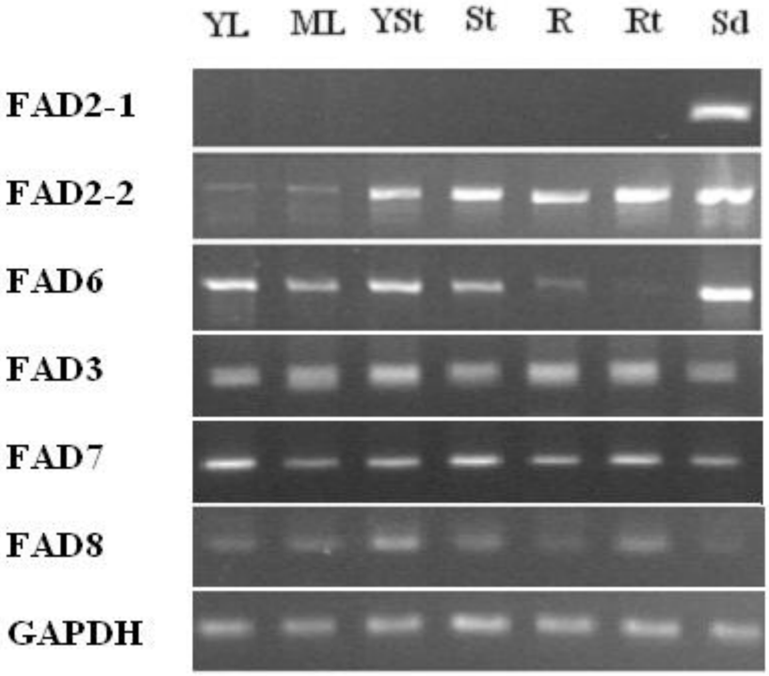


Figure 4.3.2. Differential expression of desaturase genes in different organs of *Portulaca oleracea* L. plants. YL-young leaf, ML-mature leaf, YSt- stem tips, St-stem, R-root, Rt-root tip, and Sd–seed.FAD – Fatty acid desaturase gene.

Delta-12 fatty acid desaturase of *Arabidopsis* is encoded by a single *FAD2* gene in all kinds of tissues while there are two different cDNA sequences in soybean, *FAD2*-1 and *FAD2*-2, encoding microsomal delta-12 fatty acid desaturase. The *FAD2*-1 gene is strongly expressed in developing seeds only, while *FAD2*-2 gene is constitutively expressed in both vegetative tissues and developing seeds (Heppard *et al.*, 1996). In *Olea europaea*, the desaturase encoded by *FAD2*-1 is possibly responsible for the desaturation of reserved lipids in young seeds, while the desaturase encoded by *FAD2*-2 may be involved mainly in storage lipids desaturation in mesocarp and maturing seeds (Hernandez *et al.*, 2005). In cotton and sunflower, this enzyme is coded by multiple genes expressed in different tissues (Liu *et al.*, 1999a, b; Martinez-Rivaz *et al.*, 2001; Pirtle *et al.*, 2001). Similar to *Arabidopsis FAD2*, maize *FAD2* soybean *FAD2*-2, cotton *FAD2*-2, *FAD2*-3, *FAD2*-4, and sunflower *FAD2*-2 and *FAD2*-3, *Portulaca FAD2*-2 is expressed in vegetative tissues and developing seeds (Fig.4.3.1). In the other hand, like cotton, soybean and sunflower *FAD2*-1, Portulaca *FAD2*-1 is only expressed in developing seeds (Fig.4.3.1.). Falcone *et al.* (1994) observed a progressive decrease of *FAD6* expression during later stages of *Arabidopsis* plant growth in photosynthetic tissues, which may indicate a reduced demand for membrane biogenesis in fully expanded leaves, compared with young expanding leaves. Miquel and Browse (1992) also refer that gene alterations involving *FAD6* gene were exclusively expressed in photosynthetic tissues in *Arabidopsis* plants. *Portulaca FAD6* gene was expressed in all plant tissues, except root tips (Fig. 4.3.1), including non photosynthetic tissues, such as seed and root, with higher expression present in young developing leaves and stems. *PoleFAD7* showed a dominant, but not restricted, expression in photosynthetic tissues, such as leaves and stems (Fig. 4.3.1), being also detected in root and seeds. The transcript of the *FAD7* gene was observed only in the chlorophyllous tissue of *Arabidopsis* (Nichiuchi *et al.*, 1995) and tobacco (Hamada *et al.*, 1996) plants, however other reports have indicated that *FAD7* gene expression can also be detected in non-photosynthetic tissues, including developing embryos of linseed (Lay *et al.* 1990), in maturing pollen grains of *Brassica napus* (Piffanelli *et al.* 1997), and in the flower apparatus of *Olea europaea* (Poghosyan *et al.,* 1999). Expression of *PoleFAD8* gene was detected, although at low levels in all organs, with slight higher levels in stems and root tips (Fig. 4.3.1). Purslane is a semi-tropical plant, with an optimal growth temperature of 30ºC, so growing the plant at 25 ºC may have activated the transcription of *PoleFAD8*, leading to detectable levels of accumulation in all analyzed tissues. In *Descuraina sophia* plants, *FAD8* gene expression was restricted to photosynthetic tissues such as leaves, stems and young siliques (Tang *et al.*, 2007). *P. oleracea FAD3* gene was detected in all tissues analyzed, with higher levels present in the shoot tips and roots (Fig 4.3.1). Horiguchi *et al.* (1998) also reported the preferential expression of *FAD3* genes in cells that have an ability to proliferate such as the apical meristem of both shoot and root and in embryos developing after flowering in wheat (*Triticum aestivum* L.)

The expression pattern of these fatty acid desaturase genes suggests different roles on the lipid desaturation process in purslane tissues. *PoleFAD2-1* was exclusively involved in storage lipids desaturation in seed, supported by its exclusive expression on seeds. *PoleFAD2-2* and *PoleFAD3* may be partially responsible for desaturation of storage lipids in developing seeds and also be involved in membrane lipids desaturation in root, leaf and stem. *PoleFAD6, PoleFAD7* and *PoleFAD8* genes may have a more extensive role in membrane lipid desaturation in photosynthetic tissues such as leafs and stems, but also, to a lesser extent in the seed storage lipid desaturation.

5. Effect of salinity on polyunsaturated fatty acid accumulation and ω-3 and ω-6 fatty acid desaturases gene expression in purslane (*Portulaca oleracea* L.) leaves.

Alternative sources of PUFA are desirable, and the concept of obtaining them from higher plants in commercial and sustainable quantities is particularly attractive. Purslane (*Portulaca oleracea* L.) is a rich source of lipids, with a high ω3/ω6 ratio (Kumamoto *et al.*, 1990; Omara-Alwala *et al.*, 1991; Simopoulus, 2004). The proven ability of purslane to accumulate higher amounts of ALA than LA acids in leaves makes it a valuable crop, providing a desirable vegetable source for PUFAs in the diet.

It has been described that environmental conditions modulate the ALA/LA (C18:3/C18:2) ratios of membrane and storage lipids through effects on desaturase enzyme activity and possibly gene expression (Hamada *et al.*, 1996; Zhang *et al.* 2005; Im *et al.* 2002). The identification of genes that control and facilitate the accumulation of these fatty acids in purslane leaves, and studying the way that environmental conditions affect these genes's expression, would provide useful tools for increasing the concentration of LA and ALA in plant tissues by means of either improved selection or genetic modification. This work aims at study purslane response in terms of plant yield, desirable PUFA accumulation and ω-6 and ω3 fatty acid desaturase genes relative expression, under imposed chloride-salinity conditions.

In this work, we measured the effect of chloride-salinity in plant growth and yield, ω3 and ω6-desaturase genes expression level and accumulation of linoleic (LA) and linolenic (ALA) acids, in purslane leaves (**Paper IV**). Twenty-one-days-old purslane seedlings were transplanted into a closed hydroponics system under greenhouse conditions (Fig. 5.1.). Eight plants were randomly placed in each tank and two tanks were used for each treatment. The tanks were filled with treatment/control nutritive solutions and were aerated for 15 minutes every two hours to ensure proper oxygen supply to the plant roots (Fig. 5.1.). Plants were exposed to 4 levels of chloride-salinity on the root-zone: 60, 90, 120 and 240 mM NaCl, and relationships between salinity, gene expression and ALA/LA levels were followed through the plant life cycle.

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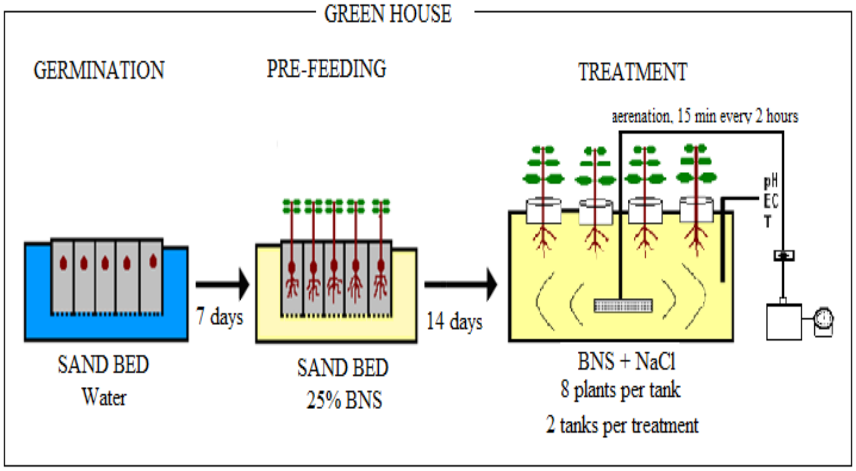


Figure 5.1. Purslane soil-less culture. Seeds were sowed in a sowing tray filled with sand and the sowing tray was placed in a water bed for 7 days. Then the water was replaced by basal nutritive solution (BNS) at 25% strength for 14 days. 21 day old seedlings were then transferred to the closed hydroponics system, were the saline treatments were applied. The applied saline treatments consisted in the addition of NaCl to the basal nutritive solution in the concentrations of 60, 90, 120, and 240 mM NaCl BNS - Basal nutritive solution (control) composition: Nitrogen at 200 µg ml-1 (NO_3^-:NH_4^+ ratio of 0.5:0.5), macronutrients (mM): 31P, 207 K, 200 Ca, 48 Mg, 64 S, and micronutrients (µM): 2 Na, 50 Cl, 24 B, 2 Mn, 2 Zn, 0.5 Cu, 0.5 Mo, and 50 Fe-EDDHA.

Although a reduction on the plant dimensions and fresh weight were observed, no chloride-salinity toxicity signs or plant death were observed in any of the treatments, an indication of this plant's high tolerance to a saline environment. Yield reduction of 10-15% was observed for 60 mM NaCl treatment; more than 50 % reduction was observed only for 120 mM and 240 mM NaCl levels, after 21 days of imposed treatment (Fig. 5.2.)

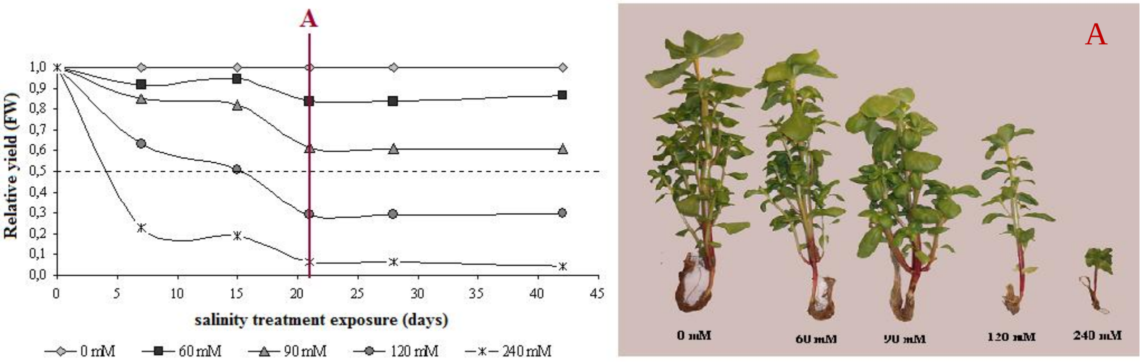


Figure 5.2: Relative Yield of purslane plants exposed to chloride-salinity treatments under 42 days of treatment at different NaCl concentrations. A – Evident reduction on plant growth after 21 days of saline treatment exposure, particularly in higher salinity levels.

In this work, we measured the effect of chloride-salinity on ω3 and ω6-desaturase genes expression level and accumulation of linoleic (LA) and linolenic (ALA) acids, in purslane leaves. Triplicates of each sample were used for the fatty acid analysis (by GC/MS) and semi-quantitative real time PCR (Fig 5.3.).

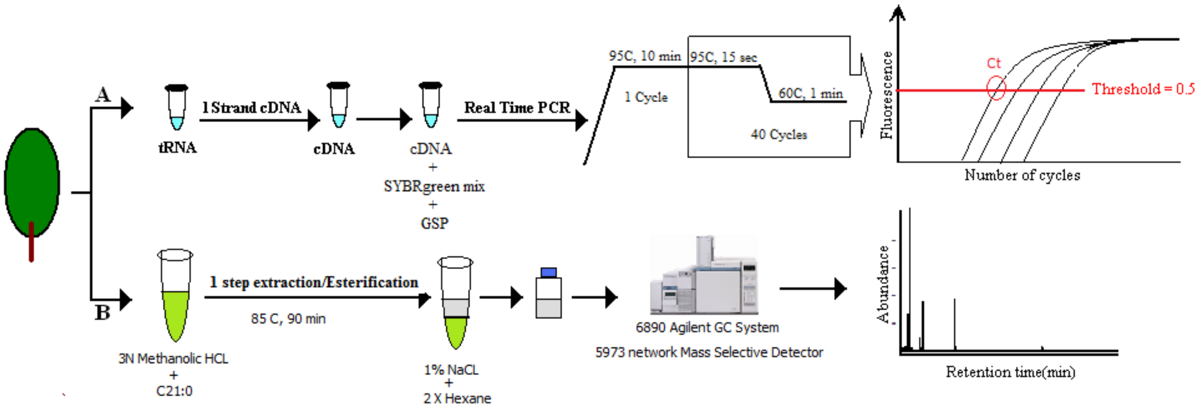


Figure 5.3: Sample analysis procedures. A- Relative gene expression determination by real time PCR. B – Fatty acid quantification by GC-MS analysis.

Total fatty acids (TFA) were extracted and esterified in 3N methanolic HCl, at 85°C for 90 min (Vriten, 2005). The reaction was stopped by adding 1% NaCl solution and methylated fatty acid were extracted with 2 volumes of hexane. The extract was evaporated to dryness in a He stream and resuspended in 1 ml hexane for GC-MS analysis. Heneicosaenoic acid (C21:0) was used as an internal standard for quantification proposals.

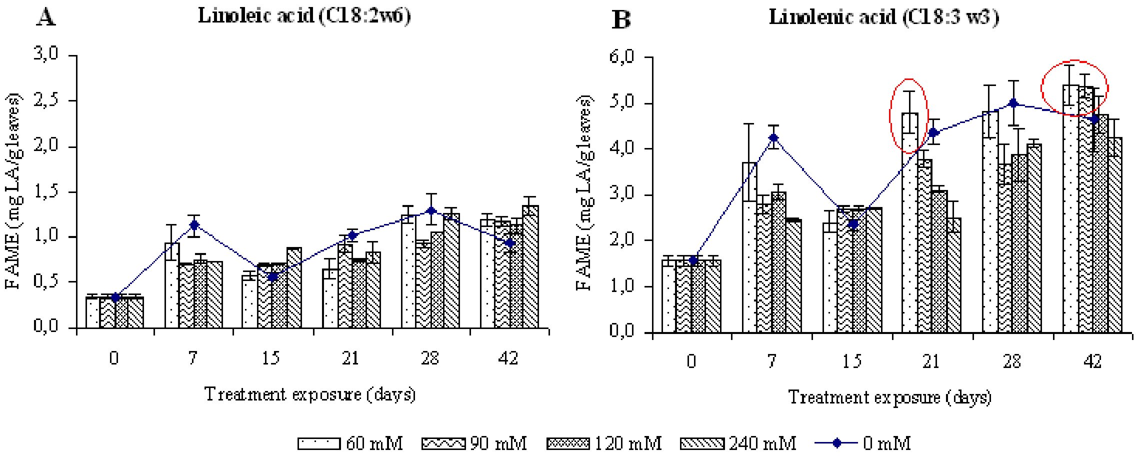


Figure 5.4. Selected fatty acid contents of purslane leaves from plants exposed to chloride-salinity during 42 days of treatment. A - Linoleic acid (LA) content, mg LA/g fresh leaves; and B- Linolenic acid (ALA) content, mg ALA/g fresh leaves. Circles indicate linoleic acid accumulation level above the values registered for the control samples at the same given time.

Table 5.1. Selected fatty acids accumulation: linoleic (LA), and linolenic (ALA) content in purslane leaves (mg FAME/ g fresh weigh); ALA/LA ratios in purslane plants after 21 days of chloride-salinity treatment.

NaCl (mM)	LA (mg/g leaves)	ALA (mg/g leaves)	ALA/LA
0	1.03 (14.6 %)	4.37 (62.7 %)	4.24
60	0.65 (12.7 %)	4.80 (63.1 %)	7.38
90	0.92 (15.2 %)	3.78 (62.3 %)	4.11
120	0.76 (15.1 %)	3.11 (62.0 %)	4.09
240	0.84 (18.6 %)	2.51 (56.0 %)	2.99

Values in brackets correspond to the proportion, in percentage of LA, and ALA, of the total fatty acid content.

Evidences on the importance of dienoic fatty acids in the tolerance to salt and drought were shown by the introduction of two sunflower (*Helianthus annus*) ω-6 desaturase genes (*FAD2*-1, *FAD2*-3) into yeast, which resulted in the production of dienoic fatty acids, an increased unsaturation index and fluidity, increased tolerance to NaCl and increased tolerance to freezing (Rodríguez-Vargas et al, 2007).

In this study, the accumulation profile of LA and ALA in the leaves was measured by GC-MS and was in agreement with the changes in gene expression level. Although an overexpression of purslane *FAD2* and *FAD6* genes or a significant increase on the LA content were not detected, LA/OA index increased with increasing NaCl levels, which may indicate an adjustment of the membrane fatty acid composition to a higher level of unsaturation.

In many plant systems, salt/drought stress results in reduced ALA. Therefore it would be reasonable to assume that this reduction is a component of their defense mechanism against the stress, rather that one aspect of the damage (Upchurch, 2008). Experiments with transgenic tobacco cells and plants demonstrated that the overexpression of ω-3 desaturases, which increases the ALA, increases tolerance to salt and drought stresses (Zhang *et al.*, 2005).

Relative expression of genes involved in LA and ALA synthesis in purslane plants exposed to different saline treatments was studied by RT-qPCR. We performed RT-qPCR experiments with cDNA obtained from total RNA samples extracted from adult mature leaves of purslane. In this work, the relative expression of two forms of ω6-desaturase genes: *FAD2* and *FAD6*, and three forms of ω3-desaturase genes: *FAD3*, *FAD7* and *FAD8*, were determined using the primer sets previously described in Table 4.3.1. The target genes relative expression value was calculate in relation to control plants, and all transcript accumulation values were normalized to Ubiquitin gene (control). The relative expression of both, microsomal and plastid forms, of the desaturase genes were influenced by the treatments and stage of plant development.

RT-qPCR analysis showed an increase of expression of ω-3 desaturase genes, *FAD3*, *FAD7* and *FAD8*, at 60 mM and 90 mM NaCl treatments at different rates, which in a certain degree was reflected by an increase of linolenic acid accumulation (Fig. 5.4), although a direct correlation between these two parameter was not established A reduction in linolenic acid and a down-regulation of omega-3 FAD genes was observed at the highest levels of treatment.

The increase of the expression of ω-3 desaturase genes, as a response to moderate salinity conditions, with subsequent increase of linoleic acid accumulation, may be an indication that the degree of insaturation in the membrane lipids composition plays a role in purslane ability to subsist in harsh environments.

6. Chilling and wounding effect on linoleic/linolenic accumulation and fatty acid desaturase gene expression in purslane (*Portulaca oleracea* L.) leaves

The chloroplast membranes of higher plants have unusually high concentrations of trienoic fatty acids, with linolenic (C18:3) or a combination of linolenic and hexadecatrienoic (C16:3) acids, making up more than 80% of the fatty acids found in this organelle (Harwood, 1988; Ohlrogge and Browse, 1995). Although the processes of acclimatization is considered to include complex and various biochemical processes, Kodama *et al.* (1995) shown that an increase in total fatty acid content, during the acclimation process was one of the important factors in chilling tolerance in tobacco leaves (Kodama *et al.*, 1995). An increase in trienoic acids, particularly C16:3, was also detected in leaves of plants grown at low temperature (15 ºC). In our work we analyzed the total lipid, linoleic and linolenic acids content in intact and wounded leafs of fully gowned plants exposed to low temperature (5 ºC) for 24h. An increase in these parameters was detected in purslane leaves of chilled and wounded plants, particularly the linoleic (C18:2) and linolenic (C18:3) acids content (Table 6.1). The increase in the insaturation level in the membrane may contribute to plant survival at low temperatures in purslane plants. We also detected an increase on linoleic and linolenic acid accumulation in wounded purslane leaves, which may be associated to the activation of octadecanoic pathway to produce JA, since linolenic acid is a known precursor for this stress-signaling molecule. The higher level of linolenic acid was detected in leaves of plants subjected to both, low temperature and wounding, but no cumulative effect was noticed (Table 6.1).

Table 6.1. Linoleic (C18:2) and linolenic (C18:3) content in purslane leaves of plants exposed to chilling and/or wounding stresses.

Treatment	Total FA (mg/g leaf FW)	PUFA (mg FA/g leaf FW)		
		C18:2	C18:3	C18:3/C18:2
C	3.41 (0.29)	0.513 (0.08)	1.99 (0.24)	3.89
T	4.64 (0.29)	0.668 (0.06)	2.74 (0.27)	4.11
W	4.30 (0.30)	0.621 (0.09)	2.68 (0.18)	4.32
T+W	6.10 (0.58)	0.799 (0.07)	3.63 (0.31)	4.55

FA- Fatty acids; PUFA- Polyunsaturated fatty acids

C –intact leaves from control plants (Kept at 25ºC), T-intact leaves from plants exposed to chilling temperature (5ºC) for 24 h; W- wounded leaves from plants kept at the same temperature as control plants, and T+W- wounded leaves from plants exposed to chilling temperatures for 24 h. Values on brackets correspond to the standard deviation values from nine replicates.

Abiotic stress-induced changes in the fatty acid composition of plant membrane lipids mainly occur through the regulated activities of fatty acid desaturases. A large body of research suggests that temperature regulates fatty acid desaturase expression at both the transcriptional and post-transcriptional levels (Upchurch 2008). In order to study the *P. oleracea* response to low temperature and wounding treatments, the mRNA transcript accumulation pattern of the two omega-6 genes: *PoleFAD2*-2 and *PoleFAD6*; and three omega-3 genes *PoleFAD3*, *PoleFAD7* and *PoleFAD8*, were followed in control and stressed plants. The expression analysis showed an increase of expression of desaturase genes in the leaves of plants submitted to the treatments compared with the control (Fig. 6.1). An increase on relative expression was detected for *PoleFAD2*-2 and *PoleFAD8* genes in chilled and wounded leaves of purslane (Fig. 6.1). No cumulative effect of chilling and temperature stresses was observed in the expression levels of the genes, but an increase in expression was also detected when both treatments were applied (Fig. 6.1). *PoleFAD6* and *PoleFAD7* mRNA transcript level were not significantly affected by chilling temperatures, but an increase of expression was detected for the wounding treatment (Fig. 6.1.).

The expression patterns of the two omega-6, *FAD2*-2 and *FAD6*, and three omega-3 FAD genes were examined by semi-quantitative real time PCR assay, using the double-stranded DNA intercalating dye SYBR Green for detection. Duplicate samples from tree different RNA preparations, each representing intact and wounded leaves of plants grown at 25 ºC and plants exposed to chilling temperature, 5 ºC, for 24 hours, were submitted to amplification. The same sets of primers, referred in Table 4.3.1, were used for amplifying the conservative cDNA fragments of the target genes and the control gene (UBI). PCRs were preformed using equal amounts of template. Great care was taken to avoid amplification of "false" signals, such as primer dimmer and unspecific products. To exclude non-specific amplifications, dissociation curves were generated and PCR amplification products were analyzed by agarose gel electrophoresis and sequencing. Absolute quantification was not important in this study and therefore only relative quantification was attempted. Relative expression of target genes in purslane tissues was calculated using the efficiency calibrated Model (Yuan *et al.* 2006). In this model, for each sample, target gene and a reference gene for internal control are included for PCR amplification from serially diluted samples. Ct number was first plotted against logarithm cDNA input, and the slope of the plot was calculated to determine the amplification efficiency (E). ΔCt for each gene (target or reference) was then calculated as the average Ct number of six distinct samples. The transcript abundance ratio of the target gene to reference gene was determined by equation 1.

Relative expression = (Eref) ΔCt ref/ (Etarget) ΔCt target   Equation 1 (Yuan *et al.*, 2006)

The RT-qPCR protocol was optimized by determining the optimal primer concentration and primer efficiency. A standard fluorescence threshold was set to a ΔRn of 0.5 on the log fluorescence scale to determine the fractional cycle number (Ct value). For each cDNA sample, relative expression levels of each protein coding gene were normalized by reference to the UBI gene assay and expressed in function of the control samples. UBI gene was selected as a reference gene for stress assays because it showed constant transcript level in all tested treatments, while GAPDH gene, showed variation in expression in the tested treatments.

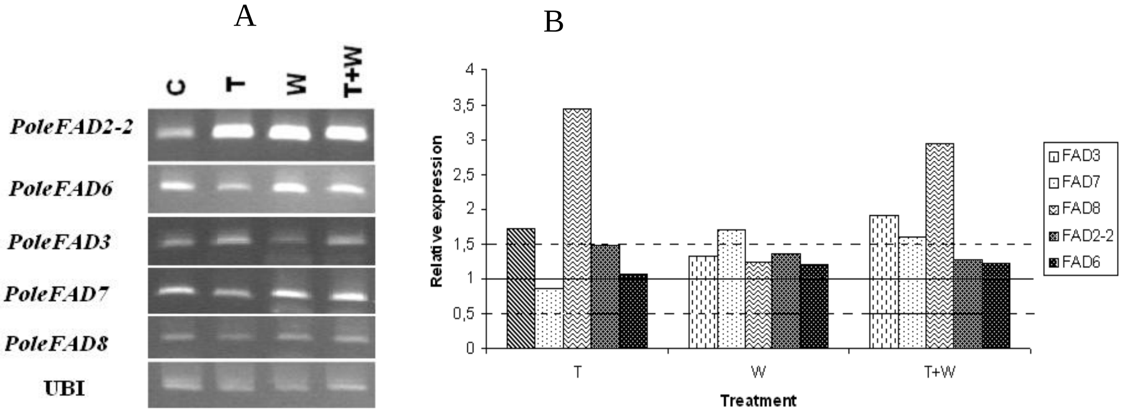


Fig. 6.1. Real-time PCR spatial analysis of *PoleFAD2*-2 and *PoleFAD6* genes in leaves of stressed purslane plants. First strand cDNAs were synthesized from total RNA extracted from intact (C) and wounded (W) leaves of plants grown at 25 ºC and plants exposed to chilling temperature, 5 ºC, for 24 h (T and T+W, respectively). **(A)** Agarose gel image of amplified *FAD* genes fragments in leaves of stressed purslane plants; **(B)** Relative expression of target genes in leaves of *P. oleracea* plants subjected to chiling and wounding treatments. The continuous line corresponds to a relative expression level of 1, in which the target gene expression from the plant exposed to the wounding and chilling treatments equals the target gene expression in the control plants. The interupted lines correspond to the upper and lower limits considered for a up- or down-regulation of each gene.

In many plant species, the content in di- and tri-unsaturated fatty acids increases under cold stress. However, the increase in polyunsaturated fatty acids was, in most cases, not attributed to a rise in mRNA levels of the enzyme. A study on *FAD2*-3 from soybean (Li *et al.*, 2006) and *Arabidopsis FAD2* gene (Okuley *et al.*, 1994) were in accordance with the general acceptance indicating that the increase in di-unsaturated fatty acid composition under cold stress is due to post-transcriptional/post translational modifications on the enzyme rather than to increases in its mRNA levels (Heppard *et al.*, 1996;. Falcone *et al.*, 2004, Tang *et al.*, 2005). However, in our study, we observed an increase in the expression of *PoleFAD2*-2 gene in response to chilling temperature and wounding stresses (Figs. 6.1 A and B.) which may indicate that an increase in mRNA levels occurred in response to the applied treatment. Similar results were reported by Kargiotidou *et al.* (2008) where an increase in mRNA levels of a membrane modifying enzyme were also detected in cotton (*Gossypium hirsutum*) plants under cold and wound-induced stresses. It was also reported that *FAD2* expression under chilling temperatures was also light –dependent.

Wounding enhanced the accumulation of *PoleFAD6* and *PoleFAD7* gene mRNA, showed by the increase in expression levels of the gene in wounded leaves of purslane in relation to intact leaves of plants grown at 25 ºC (Fig. 6.1B). An increase of expression was also detected in purslane plants exposed to both, temperature and chilling treatments. Since no significant alteration in expression levels was detected when only chilling stress was applied, we considered that the increase of expression levels when both treatments were applied was due to the wounding stress and not the temperature.

7. Heterologous expression fatty acid desaturase genes in *Saccharomyces cerevisiae* system.

In recent years many of the genes involved in synthesis of polyunsaturated fatty acids (PUFA) in plants have been identified and sequenced. The fatty acid desaturase genes (*FAD*) are well conserved and encode membrane-bound enzymes localized either in the plastid or endoplasmic reticulum (ER). Expression of plant desaturases in the yeast *Saccharomyces cerevisiae* has offered a rapid method to verify enzymatic activity of the desaturases as well as characterize their substrate/product relationships. *S. cerevisiae* has a very simple fatty acid composition dominated by palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0) and oleic (C18:1) acids, which allows detection of minute amounts of unusual fatty acids in yeast lipids. This is a crucial aspect of the heterologous expression system, since the products of the plant desaturases usually account for a very small percentage of total yeast lipids. In several cases, growth of yeast at cooler temperatures resulted in accumulation of higher amounts of PUFA synthesized by trangenically expressed plant desaturases. However, it was unknown whatever the increase in PUFA was a result of increased physiological demand for unsaturated fatty acids at lower temperatures, or whether lower temperatures had some effect on the plant enzyme.

7.1. Heterologous expression of *PoleFAD6* and *PoleFAD7* genes

For the characterization of *FAD6* and *FAD7* genes involved in fatty acid desaturation process, we transformed the *P. oleracea FAD6* and *FAD7* genes into *S. cerevisiae* using two the high-copy yeast expression vector , PYeDP60 and PYeS2, under the control of a galactose inducible promoter, GAL1, according to the procedures described by Gietz *et al.* (1992). For in vivo production of linoleic and linolenic acids, the engineered yeast was cultivated in a bioreactor minimal medium lacking uracil (SC-ura), tergitol (Type NP-40, 0.1% [v/v], and linoleic or α-linolenic fatty acids (150μM). The cells were grown in medium containing 2 % rafinose for one day hereafter expression of FADs was initiated by the addition of 2 % galactose. The cultures were induced for 24 hours and linoleic and linolenic production was quantified by GC/MS system, after cell disruption and fatty acid esterification, using hexane as a solvent. Aliquots of 5 ml of culture were collected every day, for a 15 day period. Fresh medium was added every time a harvest was conducted.

The linoleic and linolenic acids production was monitorized by GC/MS chromatogram, in either of the tested systems. The presence of the substrate indicates that the yeast cell is able to incorporate the substrate from the medium into its cell membranes, but no expected product was detected in the assays (Fig. 7.1.1).

The GC-MS analysis of fatty acid methyl esters from yeast cultures showed that the endogenous desaturation products in *Saccharomyces cerevisiae* cells, palmitoleic ($C16:1^{\Delta 9}$), and oleic ($C18:1^{\Delta 9}$) fatty acids, constituted the major fatty acids fraction. Other endogenous fatty acid like palmitic acid (C16:0), and stearic acid (C18:0) were also detected in much lower concentration. Linoleic acid ($C18:1^{\Delta 9, 12}$) was only present in cultures were it was added as a substrate in the media (Fig. 7.1.1). The fatty acid profile in all cultures did not significantly change along the assay, with the increase of incubation time. As an example, fatty acid profiles of wild type and transformed *PoleFAD6*-pYeDP60/pK113-5D and *PoleFAD7*-pYeDP60/pK113-5D (Fig. 7.1.1.) cultures with 14 days of incubation are represented.

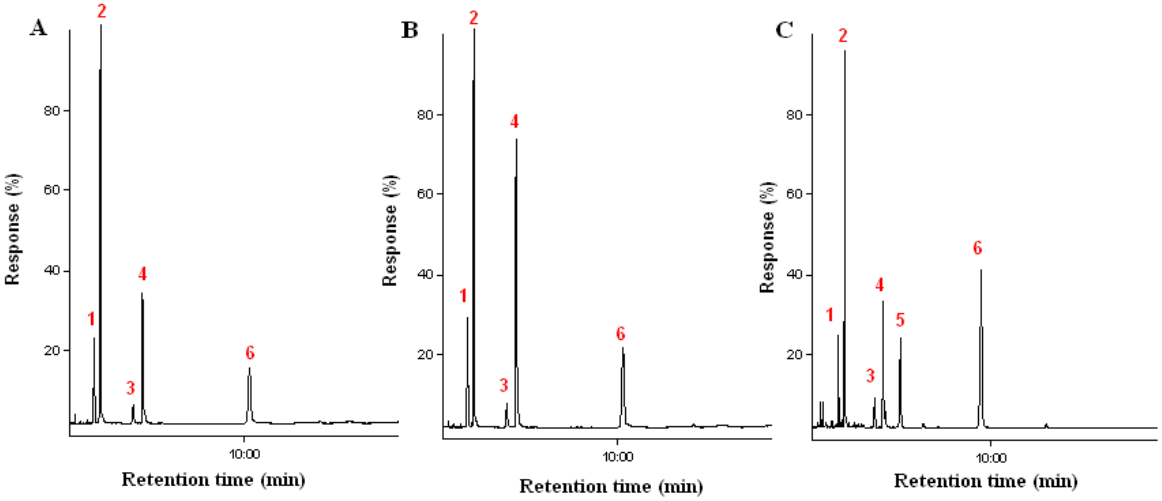


Figure 7.1.1. Gas chromatograms of fatty acid methyl esters from wild and transformed yeast cultures with the *pYeDP60* yeast expression vector carrying *PoleFAD6* and *PoleFAD7* genes: Wild (A), *TY1-PoleFAD6* (B) and *TY2-PoleFAD7* (C) at 20C; 14 days after substrate addiction. 1- Palmitic acid (C16:0); 2 - Palmitoleic acid (C16:1 Δ9); 3 - Stearic acid (18:0); 4 - Oleic acid (C18:1 Δ9); 5-Linoleic acid (C18:2 Δ9, 12); 6 - Heneicosaenoic acid (C21:0) (internal control).

The conditions of growth tested in present experiments are similar to former desaturases expression studies in yeast (Hong-mei *et al.*, 2006; Reed and Covello, 2000; Covello and Reed, 1996; Higashi and Murata, 1993). Nevertheless it was not possible to detect the activity of the cloned desaturase in transformed yeast, enabling to conclude about the ideal conditions for the expression of the *PoleFAD6* or *PoleFAD7* genes in *S. cerevisiae*. An incorrect conformation of the desaturases could also have occurred, leading to the production of a non-functional enzyme. Slight changes to the procedure have to be tested. Given that *PoleFAD6* and *PoleFAD7* were under the control of the GAL1 promoter, is unlikely that the desaturases were not expressed, since this is a strong promoter and transcription would be induced by the presence of galactose in media. Los and Murata (1998) referred that the conserved histidine clusters together with iron ions are presumed to compose the Fe-binding active centers of the desaturases, so the limitated amount of iron in the media may have affected the enzyme activity, leading to a very low product accumulation, unable to be detected in the functional assay. Further experiments will be carried out, using a fluorescent marker, a green fluorescent protein (GFP) that will be attached to the C-terminal of the target protein, in order to detect protein expression and location in the yeast cell, using fluorescent microscopy techniques.

7.1.1. Effect of iron supplementation in growth medium

The presence and availability of iron in yeast growth media may affect the expressed protein activity with higher product accumulation in the membrane, since the conserved histidine clusters together with iron ions are presumed to compose the Fe-binding active centers of the desaturases (Los and Murata, 1998). To test this hypothesis, iron supplements were added to the media and the product accumulation in yeast membranes was determined by functional assay using GC-MS analysis. To test the effect of iron concentration in the expression of *PoleFAD6* and *PoleFAD7* genes in *S. cerevisiae*, the transformed yeast *FAD6*-PYEDP60/PK113-5D and *FAD7*-PYEDP60/PK113-5D strains were cultured in minimal medium lacking uracil with 2% galactose (CM gal-ura), Tergitol (Type NP-40, 0.1% [v/v], linoleic or α-linolenic fatty acids (150μM) and iron in the form of FE-EDDHA, at 20 ºC (for rapid growth) for 3 days and at 15 ºC (product accumulation) for other three days. The iron concentration tested ranged from 1 to 4 mM Fe-EDDHA.

As previously observed in the preliminary expression assay, the major fatty acids fraction extracted from yeast cell cultures were the endogenous desaturation products: palmitoleic ($C16:1^{\Delta9}$) and oleic ($C18:1^{\Delta9}$) fatty acids. Endogenous saturated fatty acids such as palmitic (C16:0) and stearic (C18:0) acids were present in lower concentrations (Fig. 7.1.1.1.).

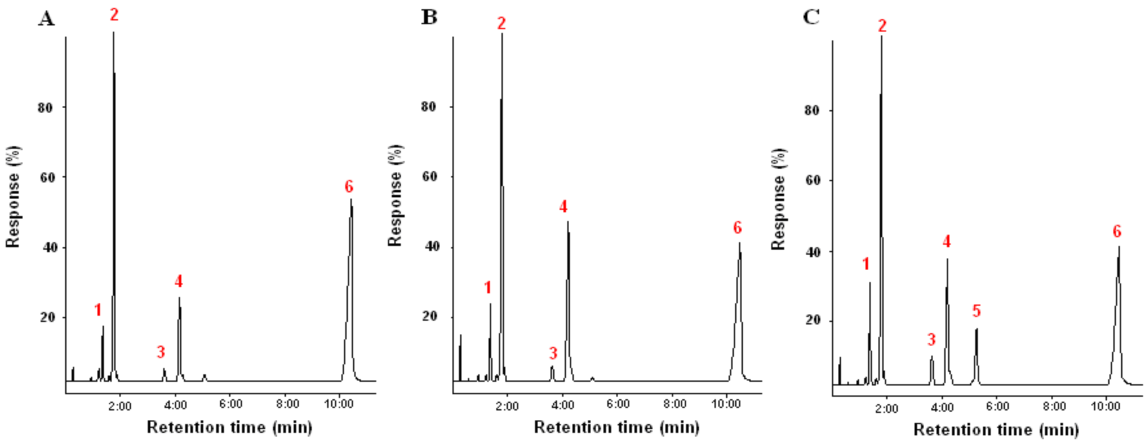


Figure 7.1.1.1. Gas chromatograms of fatty acid methyl esters from wild (A)and transformed yeast cultures with the *pYeDP60* yeast expression vector carrying *PoleFAD6* (B)and *PoleFAD7 (C)* genes cultured in media supplemented with 4 mM FE-EDDHA at 20C. 1- Palmitic acid (C16:0); 2 - Palmitoleic acid (C16:1); 3 - Stearic acid (18:0); 4 - Oleic acid (C18:1); 5 –Linoleic acid (C18:2); Heneicosaenoic acid (C21:0) (internal control).

Linoleic acid ($C18:1^{\Delta9, 12}$) was only detected in the cultures were it was added as a supplement in media (Fig. 7.1.1.1-C), which means that no endogenous linoleic product was produced. Apparently, and for the tested concentration, the iron concentration on the medium did not affect the expression of *PoleFAD6* and *PoleFAD7* genes. The wild cultures also did not show significant differences in fatty acid composition for the iron concentrations tested. Iron supplementation did not induce/increase FAD activity (coded by *PoleFAD6* and *PoleFAD7* genes) for the tested iron concentration; in fact, no activity of these proteins was detected in the functional assay.

7.1.2. Effect of pH in the target protein heterologous expression in *S. cerevisiae*

The growth conditions, such temperature, pH and nutrient availability can affect not only the yeast growth, but also the expression and activity of heterologous proteins in the yeast cells, leading to lower accumulation levels of target products.

To test this hypothesis, we grown the transformed yeast in SC medium with adjusted pH in the range of 4.5 to 8.5, and we incubate the cultures at 20 and 30 ºC. Product accumulation in yeast membranes was determined by functional assay using GC-MS analysis. We tested both genes (*PoleFAD6* and *PoleFAD7*) in both expression systems *PyeDP60/pk113-5D* and *PyeS2/INVSc1* (Vector/*S. cerevisiae* strain) and similar results were obtained. Here we refer only to the results corresponding to the use of the commercial system *PyeS2/INVSc1*.

Transformed yeast *FAD6-pYeS2/INVSc1* and *FAD7- pYeS2/INVSc1* strains were cultured in minimal medium lacking uracil with 2% galactose (CM gal-ura), Tergitol (Type NP-40, 0.1% [v/v], linoleic or α-linolenic fatty acids (150μM). The medium was buffered with 50 mM Tris-succinate (PH 4.5 -8.5).

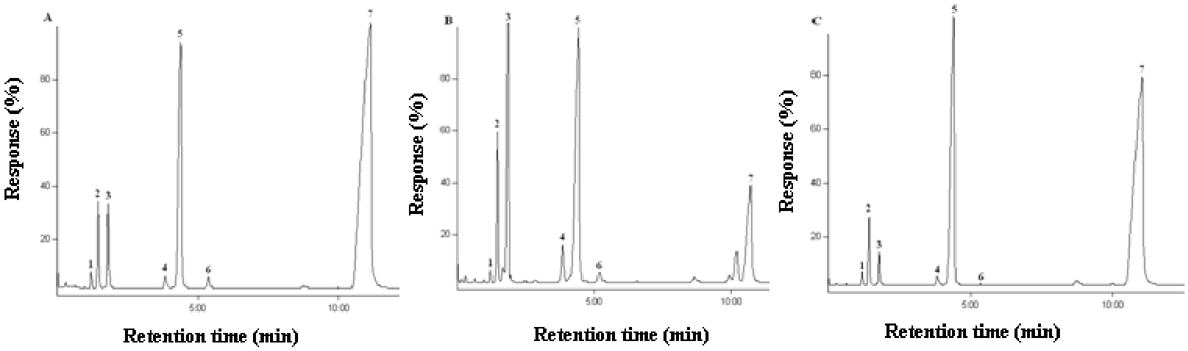


Figure 7.1.2.1. Gas chromatograms of fatty acid methyl esters from transformed yeast cultures with the *pYeS2* yeast expression vector carrying *PoleFAD6* gene cultured at different pH at 20C. A-pH=4.0; B-pH=5.0. C-pH=6.

1- Tetraenoic acid, 2-Palmitic acid (C16:0); 3 - Palmitoleic acid (C16:1); 4 - Stearic acid (18:0); 5 - Oleic acid (C18:1); 6 –Linoleic acid (C18:2); 7 - Heneicosaenoic acid (C21:0) (internal control).

The medium pH did not affect the expression of *PoleFAD7* gene and no target product was detected in any of the conducted experiments in the pH range tested, although a reduction on yeast growth was detected in the lower pH levels (pH 4.5 and 5.0). The wild cultures growth was also negatively affected by low pH values, and no significant differences in fatty acid composition were detected. No significant changes in the fatty acid profile were detected in the transformed yeast cultures, with no endogenous heterologous product synthesis being detected by the GC-MS analysis, with the exception of the PoleFAD6 clone that showed a minor, but detectable accumulation of linoleic acid at the pH range of 4 to 5 (Fig. 7.1.2.1-A,B). No product was detected for pH values equal or superior to 6 (Fig 7.2.1.1.-C) or, for the cultures incubated at 30 ºC. It seems that low temperatures enhance the production and accumulation of polyunsaturated fatty acids in the yeast cell. These data indicate that *FAD6* gene is being heterologous expressed in yeast, and the correspondent enzyme, Δ12- fatty acid desaturase is being produced and accumulated in a functional form at pH values inferior to 6.0. In the case of *FAD7* gene, either the enzymes are not being expressed or are expressed in a non functional way, since no target product was detected in the functional assay for the range of pH tested. It is also possible that the target protein is not being accumulated in the membrane but in a plasmid inside the yeast cell. In that case the protein may be present in the form of aggregates and not be in a functional form.

7.2. Sub-cellular localization of heterologous protein in yeast cell: Fluorescent Assay

An increase of iron concentration in the medium or pH value did not affect the expression levels of *FAD7* gene, so it became necessary to develop an assay that will allow to access if the protein is being expressed. To confirm if the protein is being synthesized by the yeast cells and were it is accumulated in cell organelles, a small fluorescent protein (approximately 240 amino acids) was inserted to the C-terminus of FAD gene, using a short linker of three amino acids with a restriction enzyme site in it (Fig 7.2.1). *S. cerevisiae* strain *INVSc1* was transformed with the vector caring the FAD-GFP construct. Two expression vectors were tested: pYeDP60 and pYeS2. A putative clone of each construct was sent to sequence to verify if the clone was in frame. If the translation of the gene is successful and the protein is synthesized, fluorescence will be present in the yeast cell organelle whereas the protein accumulates.

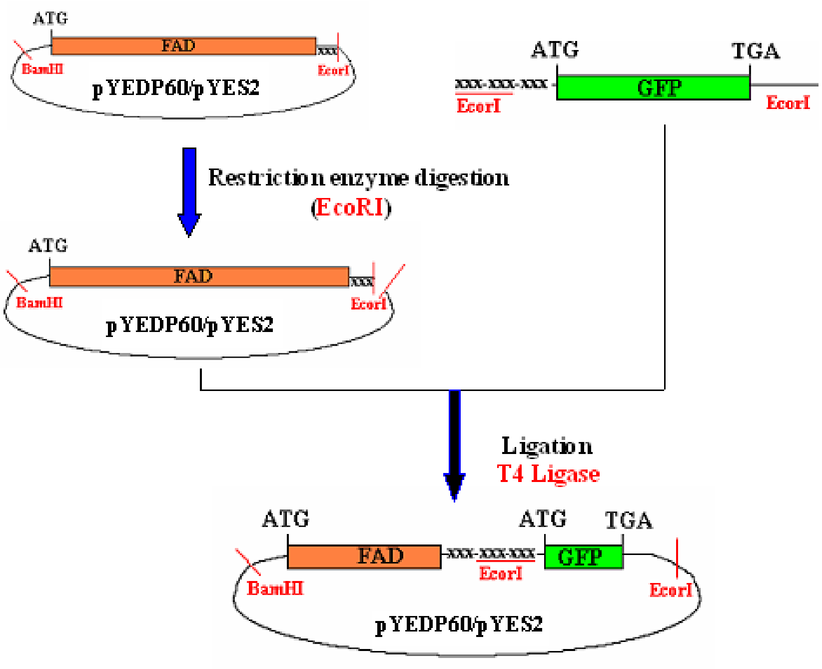


Figure 7.2.1. Squematics of construct FAD-GFP synthesis

A miniprep culture (3 ml of SC-G418 medium + 2 % galactose) of a single colony of a positive clone of each of the constructs: *PoleFAD6*-GFP (in pYeDP60 and pYES2), *PoleFAD7*-GFP (in pYeDP60 and pYES2); GFP (in pYeDP60 and pYeS2), *PoleFAD6* and *PoleFAD7* as positive and negative controls, respectively, were prepared. The cells were cultivated in a growth chamber, at 30ºC, with agitation (200 rpm) overnight. An aliquot of 10 µl of each culture was fixated into the microscope slide with 10 µl of a 1% agarose solution (warm) and the preparations were subjected to contrast microscope analysis, using blue light filter to detect fluorescence in yeast cells (Fig. 7.2.2.-B). The rest of the culture was used for the determination of the fatty acid profile by GC-MS analysis in order to access if the target fatty acids were being synthesized (Fig.7.2.2.-A).

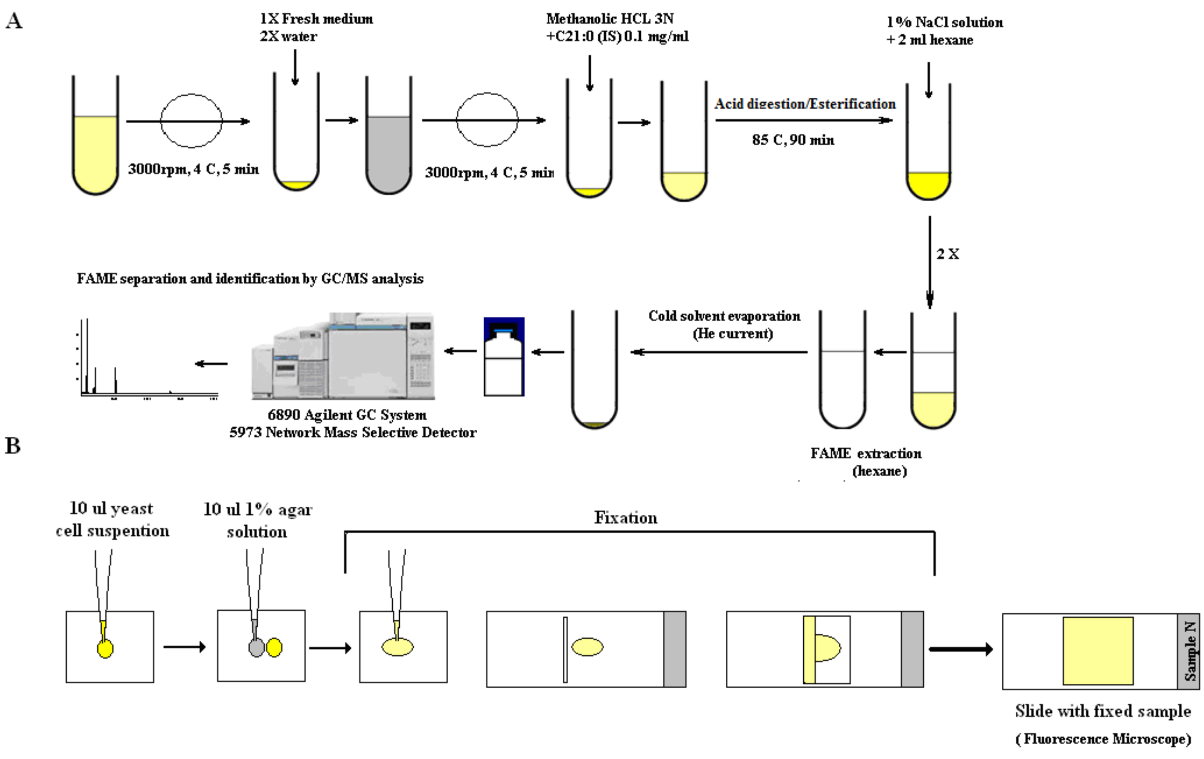
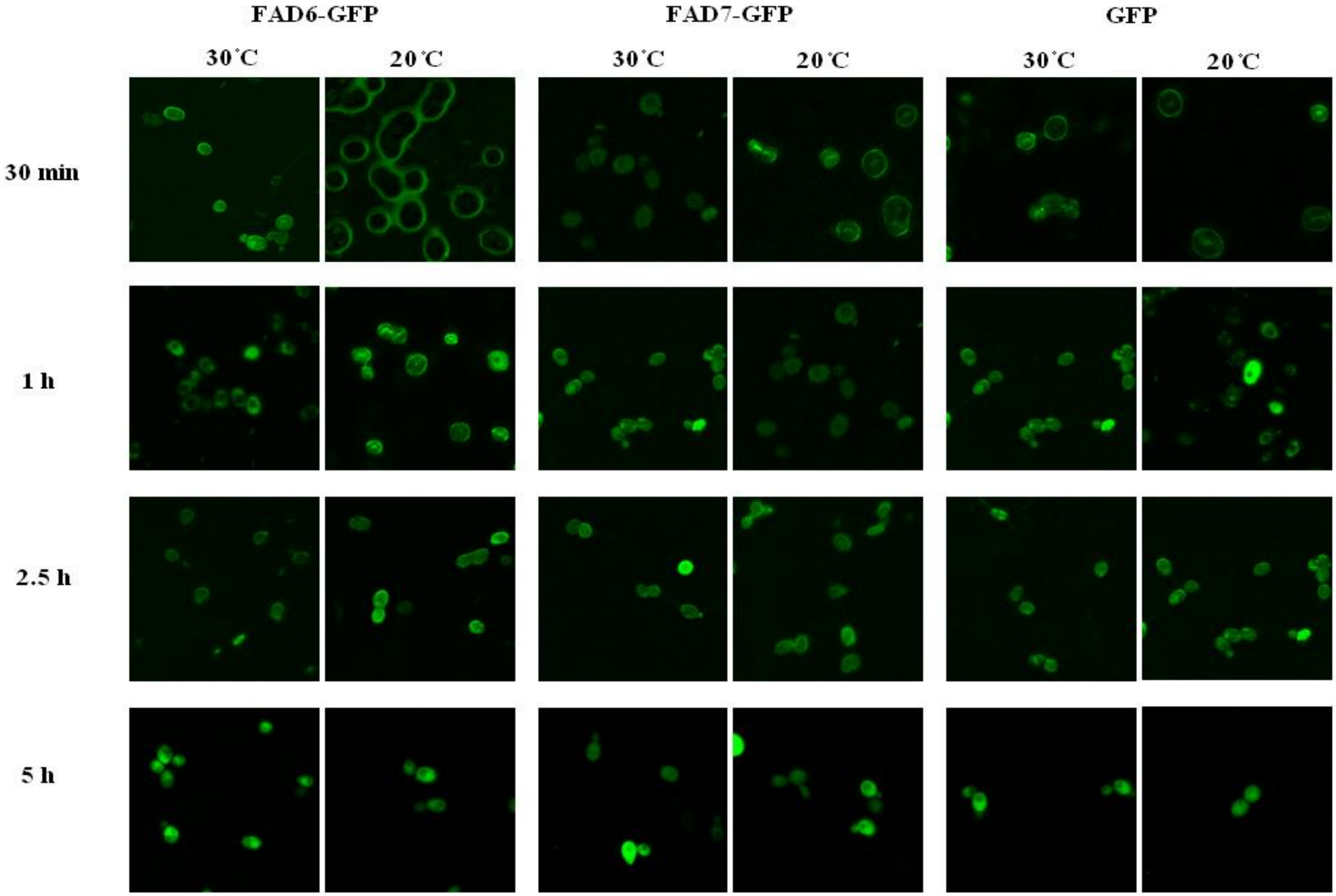


Figure 7.2.2. Analysis of the heterologous expression of *PoleFAD6* and *PoleFAD7* purslane genes in yeast system. (A) Functional assay by GC/MS for the detection of desaturation products, linoleic and linolenic acids (Vriten et al., 2005); (B) Localization of target protein accumulation organelle in yeast cell by fluorescent in differential microscopy analysis.

Preliminary testing was performed in the overnight cultures (16 h incubation). No fluorescence was detected in the negative controls, and some fluorescence was present in the positive controls. The *PoleFAD6*-GFP and *PoleFAD7*-GFP construct putative positive clones tested so far exhibited some fluorescence but in a low intensity. The fluorescence pattern was similar to the one observed in the yeast cells transformed with the GFP gene only. It was not possible to see a predominant accumulation of the protein in the membrane, as expected, and some fluorescence was also visible inside the cell, possibly in areas were the protein is being synthesized and degraded.

These results indicate that the target protein was expressed but it was not possible to locate the accumulation region within the cell. Cultures with lower incubation period were tested in the fluorescent assay, to verify if a transient accumulation of the target protein in the membranes occurs. Since the target protein is a heterologous protein, not indigenous to the yeast cell, it is possible that initially the target protein accumulates in the membrane (the target organelle) and then be mobilized and accumulated in a vacuole within the yeast cell. We decided to repeat the assay, using the commercial *S. cerevisiae* strain *INVSc1* transformed with the pYeS2 vector carrying the constructs *PoleFAD6*-GFP and *PoleFAD7*-GFP, and GFP. We analyzed the fluorescence after 0.5, 1, 2.5 and 5 h of induction, to access if a transient accumulation of the target protein the membranes was detected. In Fig 7.2.3. are represented the results obtain in the fluorescence assay, for the localization of the accumulation site of the target proteins within the yeast cell.

Figure 7.2.3. Target protein detection in yeast cells by fluorescence assay. Fluorescence pattern of *INVSc1 S. cerevisiae* strain transformed with *pYeS2* vector carrying *FAD6-GFP* construct, *FAD7-GFP* construct, and *GFP* gene (positive control) incubated at normal (30ºC) and low (20ºC). Samples were taken after 30 min, 1 h, 2.5 h and 5 h after induction with galactose.


FAD6-GFP
FAD7-GFP
GFP
30˚C
20˚C
30˚C
20˚C
30˚C
20˚C
30 min
1 h
2.5 h
5 h

From this assay it was possible to observe that up to the first hour after induction, the target proteins are being accumulated preferentially in the cell exterior membrane (Fig. 7.2.3.) for both target genes. Culturing the yeast at 20 ºC seem to induce higher rates of protein accumulation, illustrated by more intensive fluorescence (Fig 7.2.3).The GFP accumulation pattern is similar in the first 30 minutes after induction, but after one hour is possible to observe the protein being accumulated in other locals in the cell, illustrated by a diffused fluorescence in all cell (Fig. 7.2.3). After 2 hours, although a preferential accumulation of the target proteins in the cell membrane is still occurring, we also find it in other cell organelles, with a distribution pattern similar to the one observed for positive control, whereas only the GFP protein is expressed (Fig 7.2.3). It is possible that this mobilization of the protein and accumulation in other organelles affects the conformation of the enzyme, leading to the formation of protein aggregates and/or non-functional enzyme configurations that could explain the absence of desaturation product in the yeast. It is also possible that the amount of produced target molecule is very low compared to the other native fatty acids and the detection method used is unable to detect it in the crude extract.

So the question remains why is not possible to verify its activity by the functional assay? Is it low expressed in yeast cell and/or is expressed in a non functional form? Or the protein is not being accumulated in the right cell organelle (membrane), which could affect its activity?
More assays will be conducted to try to answer to these questions and obtain a functional form of these enzymes in the near future.

7.3. Conclusions

At this point we were enabling to observe any activity of the plastid desaturases (encoded by the *PoleFAD6* or *PoleFAD7* genes) in *S. cerevisiae*, although alternative growth conditions had been and are being presently tested. The iron concentration in the media, for the range of concentration tested did not seem to affect the expression of the genes, and no activity of the corresponded enzymes was detected in the functional assay. Endogenous production of linoleic acid was detected at low pH values (4 to 5), in the cultures transformed with *PoleFAD6* gene. On the other hand, medium pH had no influence on *PoleFAD7* gene expression, and no activity of the corresponded enzyme was detected in the functional assay. So far, the results suggests that either the fatty acid desaturase gene have a very low expression level or non –active forms of the fatty acid desaturases enzyme is being express. Another possibility is that the enzyme is being synthesized in an incorrect conformation, which may compromise the catalytic activity of the enzymes, leading to the production of a semi or non-functional enzymes. Fluorescence assay indicated that the protein is being expressed and that was mainly accumulated in the membrane. Fluorescence intensity levels in the transformed cells with the constructs *PoleFAD6*-GFP and *PoleFAD7*-GFP was relatively low, which may indicate low level of protein expression. At this point no conclusion can be drowned about the optimal expression conditions for these genes.

8. Towards in vitro production of polyunsaturated fatty acids: *Portulaca oleracea* L. cell culture

PUFAs are important components of the plasma membrane of the cell, where they may be found in such forms as phospholipids. PUFAs are necessary for proper development, particularly in the developing infant brain, and for tissue formation and repair. PUFAs also serve as precursors to other molecules of importance in human beings and animals, including the prostacyclins, eicosanoids, leukotrienes and prostaglandins. At present, arachidonic acid (ARA, C20:4 omega 6), eicosapentaenoic acid (EPA, C20:5 omega 3) and docosahexaenoic acid (DHA, C22:6 omega 33) are considered the most important long-chain polyunsaturated fatty acids (LC-PUFAs). Although oils derived from fatty fish like herring, mackerel, sardine or salmon contain DHA and EPA these oils are often unsuitable for human consumption or for inclusion in infant formula. Therefore other sources, e.g. microbial PUFA producers, are now actively being sought for commercial large scale production of DHA-containing oils (Wu *et al.*, 2005). Recent studies have been focus in the production of these long-chain fatty acids in *algae* (Wen and Chem, 2003; Reis *et al.*, 2003, Jiang and Gao, 2004) and plant cell cultures of bryophytes, such *Marchantia polymorpha* (Chiou *et al.*, 2001). The majority of higher plants are unable to synthesize these fatty acids, but they can synthesize and accumulate variable amounts of their precursors, linoleic and linolenic acids. Linoleic (LA) andα.-linolenic (ALA) acids are considered essential fatty acids, since they can not be synthesized by mammals and have to be obtained from diet. LC-PUFAs can de novo be synthesized from the parent essential fatty acids linoleic (18:2 omega 6) and alpha-linolenic (18:3 omega 3) acids by desaturation and chain elongation.

Worldwide there is an increasing scientific and commercial interest for development of products and technologies focused on economic PUFA production system. A need exists for oils containing higher relative proportions of and/or enriched in specific PUFAs and, therefore, new, reliable and economical viable methods of producing specific PUFAs must be developed. A plant cell suspension system can be used to produce linoleic and linolenic acids in a controllable, reliable and natural system, by altering growth conditions, without the need to use recombinant methods of production. This study aims towards the production of linoleic and linolenic fatty acids in liquid plant cell culture. No genetic manipulation is involved in this process, since these structures are obtained from the leaves of a plant that naturally is rich in these fatty acids. So the use of this system would not raise problems in public opinion. Preliminary studies showed that this system could be economically competitive with the microbiological systems with genetic recombinant expression of these enzymes.

8.1. Establishment of P*. oleracea* cell culture lines

In this project we have developed cell culture lines for the in vitro production of PUFAs by non transgenic plant cell cultures. The plant material chosen for this work is purslane (*Portulaca oleracea* L.), an important vegetable in southern Europe, Mediterranean countries and Asia. Interest in its cultivation as a food crop has increased since its identification as a rich source of ω3 polyunsaturated fatty acids (PUFAs) and anti-oxidants. Average linoleic acid content in purslane (*Portulaca oleracea*) leaves is about 1.87 mg LA/g FW (18% of total lipid), and 4.51mg/g FW linolenic acid (43%), which is higher than the amounts produced in cell culture system, but cell culture provides a controlled, reliable system that provides sufficient plant material in only 4 weeks time (opposing to the plant that requires 6 to 8 weeks to reach adult stage when higher levels of LA and ALA are obtained). Explants from one plant can originate a large number of the structures cultivated in cell suspension, which also is able to grow and multiply under these conditions. Also, this plant is considered a semi-tropical plant that requires temperature of 25-30ºC and long periods of light to grow, which enables the establishment of this culture in more cold countries or it requires the use of glass greenhouses, which would increase production costs. Suspension cell cultures are maintained in sterile conditions, under controlled and reproducible conditions of light, temperature and surrounding atmosphere, being able to be produced and maintained in any facility with an incubation chamber.

Purslane seeds were surface disinfected to remove microorganisms, germinated and grown in vitro into seedlings on solid basal MS medium (Murashige and Skoog, 1962), solidified with 0.6% (w/v) Difco purified agar. All explants were taken from this in vitro culture. *Callus* induction was tested from several plant organs. *Callus* initiation and maintenance medium consisted of MS medium supplemented with 2% (w/v) sucrose and 0.6% (w/v) Difco purified agar. All tested media were supplemented with vitamins and growth regulators. Benzyladenin (BA), 2, 4-dichlorophenoxyacetic acid (2, 4-D), Kinetin (K) and naphthalene acetic acid (NAA) were used as growth regulators, and were added to the media at different ratios. A combination of 24 different media was tested, using auxins and cytokines concentration in the range of 0 to 1 mg l-1. Explants, 2-3 mm in length, were taken from 1-month-old in vitro-grown plants and were placed on solid MS medium supplemented with combinations of growth regulators. *Callus* tissues were removed from the explants and sub-cultured at 3-weeks intervals onto the same media upon which they were initiated. In general it was observed that this plant has high regeneration properties, when placed in culture medium without hormones, the whole plant could be regenerated from these structures rather fast. This indicates that *P. oleracea* can be a good plant system for recombinant production of other interesting molecules with commercial value.

In the media supplemented only with auxins (NAA or 2,4D), stem explants were able to regenerate leafs and root in the tips of the explants (Fig 8.1. F), and leaf explants regenerated roots in the same conditions (Fig 8.1 E). In the medium supplemented with only cytokinines (BA or kinetin) the leaves developed a green compact embryo-like *calli* (8.1. A, C) and the stems regenerated leaves (Fig 8.1 B, D). No root development was observed, which may indicate that auxins are essential for the whole plant regeneration.

Four different structures were formed in solid media supplemented with different proportions of auxins/cytokinines: soft white/green friable *calli* (WFC/GFC), compact green embryo-like *calli* (CGEC) and red leafs efflorescence (RLE). The red coloration on the regenerated leafs may be due the accumulation of betalains, which this plant produce and accumulate in high amounts in the stems, resulting in the reddish-like coloration of this organ in the plant. High NAA/BAP ratios induced the development of friable *calli* (GFC) and low NAA/BAP ratios resulted in partial tissue differentiation, with the development of GCEC and RLE (Fig 8.1).

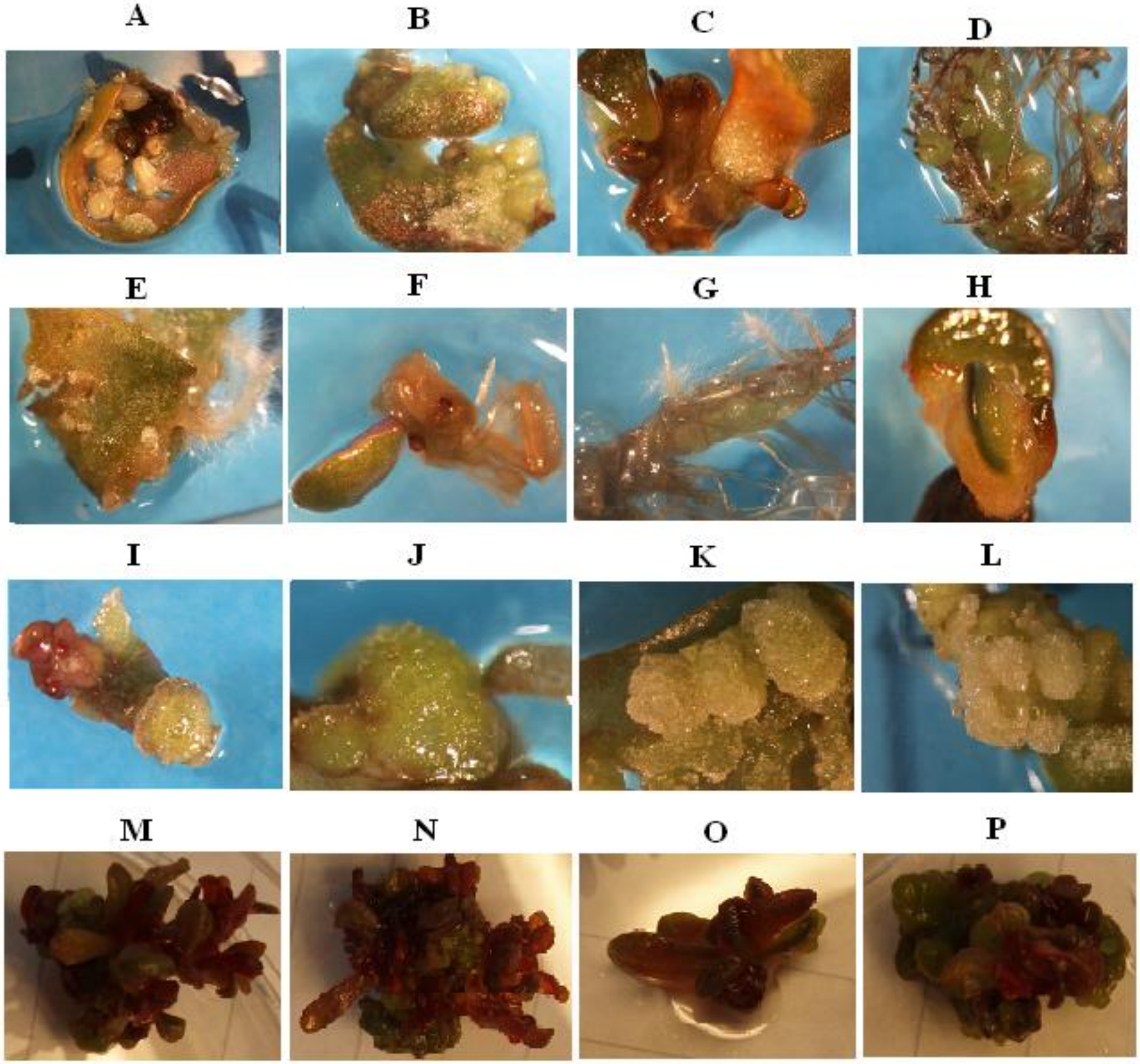

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Figure 8.1. Four week-old leaf, root and stem cultured in Ms medium supplemented with different phytohormones. Compact embryo-like structures formed in leafs cultured with MS medium supplemented with kinetin (A) and BA (B); Leaf-like structures and compact *calli* developed from stem (C) and root (D) explants cultured in BA supplemented MS medium; Root development from leaf (E), stem (F), and root (G) explants cultured with MS medium supplemented with NAA; Green friable *callus* development and leaf regeneration from stem explants cultured in media supplemented with NAA/kinetin (H) and NAA/BA (I) hormones; (J) -Green friable *callus* development from leaf explants cultured in media supplemented with NAA/BA hormones; Green friable *callus* development from leaf explants cultured in media supplemented with NAA/BA (K) and NAA/kinetin (L); Red leaf efflorescence developed from stem explants from a 8 week culture cultured in medium supplemented with 2,4D/kinetin (M), 2,4-D/BA (N), NAA/Kinetin (O), and NAA/BA (P).

8.2. *In vitro* production of PUFAs in *P. oleracea* liquid cell culture

To evaluate which tissues would be more suitable for in vitro PUFAs production, we analyzed the fatty acid composition of the four main structures formed in solid media by GC-MS analysis: white friable *calli* (WFC), green friable *calli* (GFC), green compact embryo-like *callus* (GCEC) and red-leaf efflorescence (RLE). The fatty acid profile was determined by GC/MS analysis (Fig.8.2.2) to evaluate the possibility of using these structures for in vitro fatty acid production. The original structures grown in solid media cultures were used for GC-MS analysis. In Figure 8.2.1. is represented a resume of the procedures applied in the fatty acid analysis of these samples. Approximately five grams of fresh plant material were macerated in a mortar with liquid nitrogen, and immediately used for fatty acid analysis. The one-step extraction-esterification procedure hereby applied was based on the protocol referred by Vrinten et al (2005).

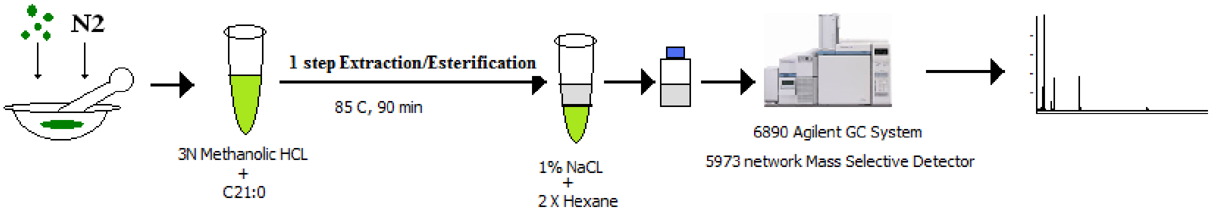

Figure 8.2.1. Fatty acid analysis procedures.

Fatty acid profile was similar between the studied structures. Palmitic (C16:0), palmitoleic (C16:1), Stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) acids and traces of arachidic (C20:0) and behemic (C22:0) were detected in the cell cultures. Polyunsaturated fatty acids fraction (C16:1, C18:1, C18:2; C18:3) corresponded to 75-80% of total fatty acids (TFA), with the exception of WFC and GFC in which palmitoleic (C16:1) and oleic (C18:1) acids constituted the main fraction of PUFA (60 and 25 % TFA, respectively) and low levels of linoleic acid (inferior to 6 %) were present. α-linolenic acid was not detected in WFC and it was detected in trace values in GFC cell cultures. Some of the desaturase are localized in the chloroplast membranes; it is possible that tissue morphological and physiological differentiation is necessary for PUFA biosynthesis. GFC and GCEC profiles were similar, with a higher accumulation of linoleic acid (30% TFA) than α-linolenic (10-15 %TFA). This result may indicate that tissue differentiation and illumination are necessary for α-linolenic acid production. Due to these results we access that WFC are not fit to be used for the production of PUFAs in suspension cell cultures, at least not on these conditions. It is well known that morphological differentiation and biochemical processes are often linked in plant cell cultures. For many species, the synthetic capacity of non-differentiated cell was lower than that of the fully differentiated tissue, both quantitatively and qualitatively. Chemical gradient in differentiated tissue complexes or *callus* aggregates encourage the synthesis of secondary metabolites (Dörnenburg and Knorr, 1995). To access if CFC, GCEC or RLE cell cultures could be used for *in vitro* PUFAs production, 1-2 g of each of these structures was transferred to liquid MS medium supplemented with the same phytohormones where they were developed, and cultured for 4 weeks in Kühner Shaker, 1SF-1-W model (Switzerland) regulated for 16h light period, at 26 °C, with smooth agitation (120 rpm).

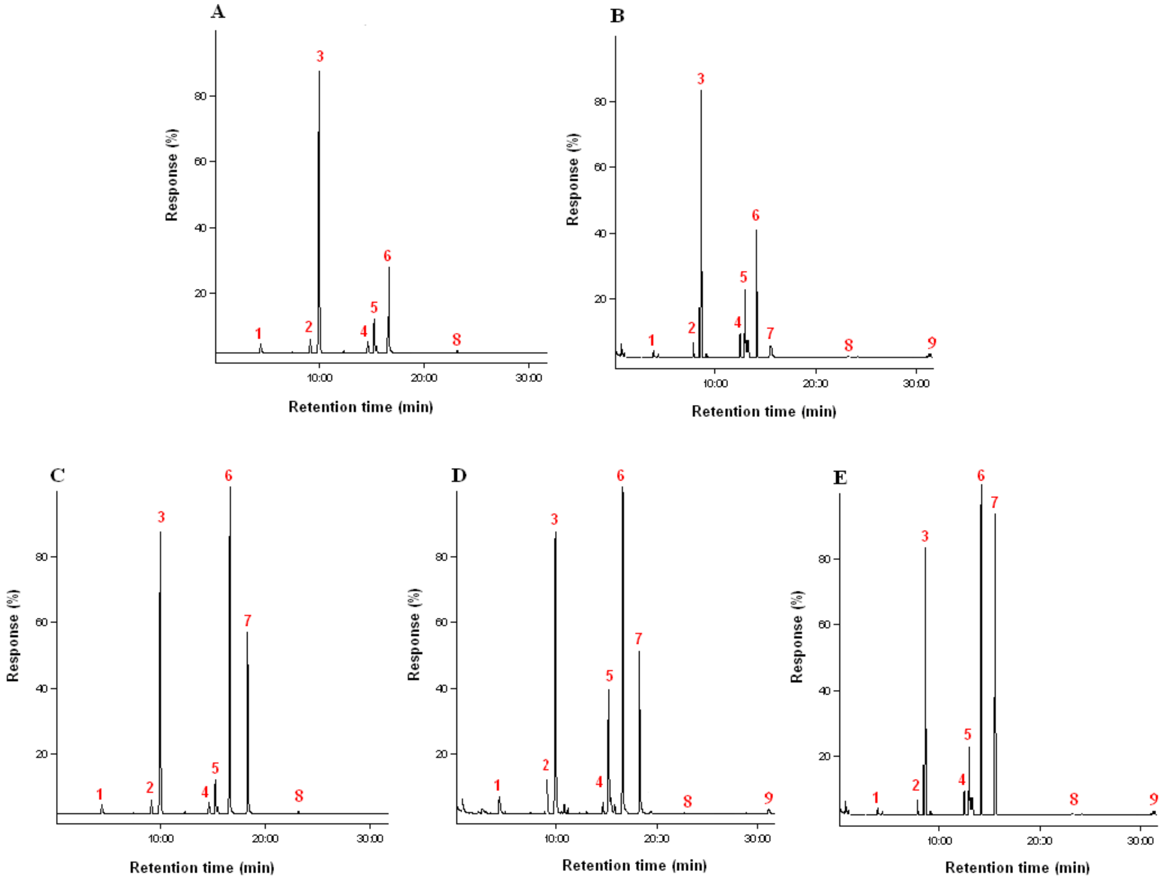

Figure 8.2.2. Fatty acid profile of *P. oleracea* cell cultures cultured in solid and liquid MS medium supplemented with different NAA/BA concentrations. (A) WFC in solid medium; (B) GFC in solid medium. (C) Semi-differentiated GCC originated from GFC in liquid culture. (D) GCEC in liquid culture, and (E) RLE in liquid culture. 1-Tetraenoic acid, 2-Palmitic acid (C16:0), 3-Palmotoleic acid (C16:1), 4-Stearic acid (C18:0), 5-Oleic acid (C18:1), 6-Linoleic acid (C18:2), 7-Linolenic acid (C18:3), 8-Arachidic acid (C20:0), 9-Behemic acid (C22:0).

No significant changes to the tissue morphology or fatty acid profile were observed in the cell cultures cultivated in liquid medium, compared to the original structures maintained in solid medium, with the exception of the GFC, which formed oval green aggregates with a semi-differentiated structure (GCC) that was able to accumulate linolenic acid (Fig. 8.2.2-C). Although, the highest amount of α-linolenic acid was present in RLE (Fig. 8.2.2-E)as it was previously observed in the solid medium cultures, maintenance in liquid culture was difficult due to the large aggregate structures formed that accumulated in the bottom of the flask, which would make impossible to keep this culture in suspension. GCC and GCEC profiles were similar, with a higher accumulation of linoleic acid (Fig. 8.2.2-C, D, respectively). Due the rapid proliferation and biomass production shown by GCC, we decided to use them as starting point for fatty acid production in liquid media.

8.3. Effect of NAA/BAP ratio in biomass production and PUFA accumulation *in P. oleracea* liquid cell cultures

We tested different NAA/BAP ratios in medium in order to optimize linoleic and α-linolenic synthesis and accumulation. The GFC grown in large scale production in liquid media and new semi-differentiated structures (GCC) with a diameter variation of 0.1 to 1.2 cm were developed (Fig. 8.3.1) in all tested combinations of NAA/BA hormones. In the CIM4 medium, with a 1:10 NAA/BAP ratio, we obtained the largest structures with 1-2 cm diameter and a dark green color (Fig. 8.3.1). CIM2 (1:1 NAA/BAP) and CIM3 (1:2 NAA/BAP) produced structures with similar sizes, approximately 0.5 cm diameter, but different color; CIM3 produced bright green structures and CIM2 produced light green structures (Fig. 8.3.1). The medium that produced the smaller structures (0.1 cm diameter) was the CIM1, whereas NAA/BAP ratio was 2:1. This data suggest that the hormone proportion in medium affects the development of these structures, and that higher auxins values negatively affect the dimension of the formed structures.

Table 8.3.1 – Characteristics off different *P. oleracea* liquid cell cultures established from leaf explants during active growth phases up to 60 days after inoculation.

Medium	Inocculum (mg DW l-1)	Water content (%)	Total biomass (g DW l-1)	Compact *callus* average size* (cm)	Compact *callus* color*
CIM1	50	95.3	3.1	0.1-0.2	White-yellow
CIM2	50	96.2	7.4	0.3-0.5	Light green
CIM3	50	97.8	8.5	0.7-1.0	green
CIM4	50	98.0	13.0	1.0-1.3	Dark-green

* See Figure 8.3.1

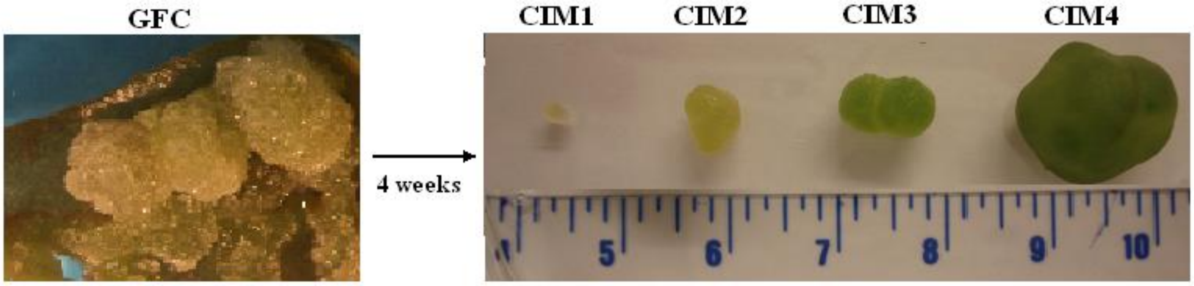

Figure 8.3.1. Four week old cultures with different NAA/BAP proportions: CIM1 (2:1), CIM2 (1:1) CIM3 (2:1) and CIM4 (1:10). GFC – Green friable *callus*

Parallel to the biomass assessment we also determine the fatty acid profile of these formed structures by GC-MS analysis. Samples of each culture (2.5 g FW) were taken every week and fatty acid accumulation was determined in dry *calli* (24h, 50 ºC). Palmitic (C16:0), linoleic ($C18{:}2^{\Delta 9,\ 12}$) and α-linolenic ($C18{:}3^{\Delta 9,\ 12,\ 15}$) acids constituted the major fraction of fatty acids present in the cell cultures (Figs. 8.3.2 A-C). Stearic (C18:0) and oleic ($C18{:}1^{\Delta 9}$) acid were also detected, but at very low concentrations (Figs. 8.3.2 A-D).

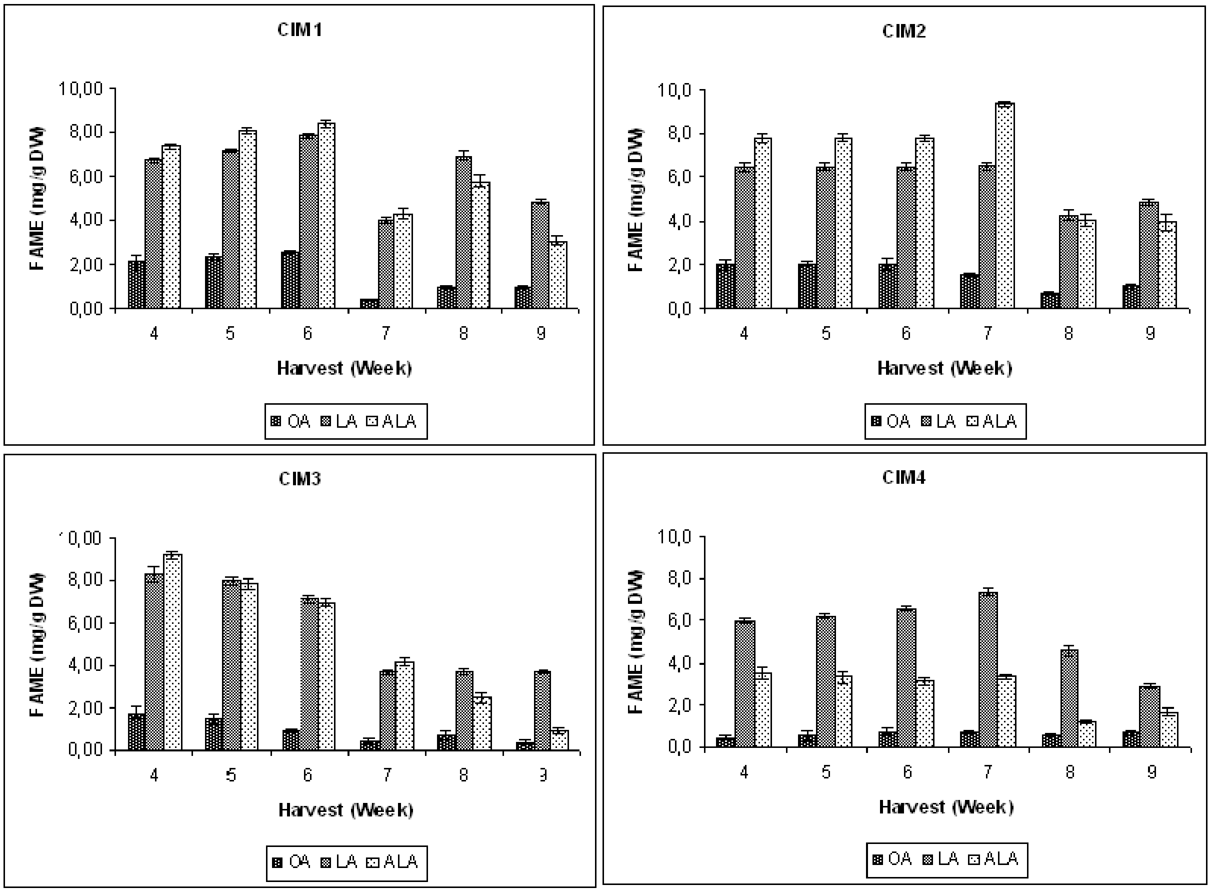

Figure 8.3.2 Fatty acid accumulation in GCC liquid cultures, in dry weight basis. OA- Oleic acid, LA – Linoleic acid, and ALA- α-linolenic acid.

The cultures maintained in MS medium with higher NAA/BAP showed higher level of linolenic acid accumulation (Fig. 8.3.2), but lower levels of biomass production (Table 8.3.1). Both LA and LNA levels decreased after 6 to 7 weeks in all cultures. The medium with the lowest NAA/BA (1:10) produced the largest structures with the darkest color, but with lower LA and ALA content. This data may indicate that, although NAA negatively affect the biomass production, it is essential for LNA production.

These results suggest that that the CIM2 medium should be used for the in vitro production of linoleic and α-linolenic acids, and that the target period for harvest should be between 4 and 7 weeks. CIM1 medium provides the culture with the highest ALA/LA ratio but in terms of biomass and culture time, the CIM2 medium offers better results. Further testing must be done to obtain oils with pure linoleic or α-linolenic acids.

8.4. Conclusions

The full processes from seeds to the development of *calli* takes about 6-8 weeks, but once the *calli* cultures are established the processes goes fast. 0.5 grams of *calli* can originate between 150 to 200 g of GCC (fresh weight) in four or five weeks. Circa 3-5 % of total biomass weight is oil, and from that more than 60 % are polyunsaturated fatty acids, particularly linoleic (LA, ω6) and α-linolenic (ALA, ω-3) fatty acids. The amount of LA is at least 3 mg/g fresh tissue, and the amount of ALA is at least 1 mg/g fresh tissue. Because of the observed high growth rate leading to good biomass production, these structures are under evaluation as good sources of plant omega-3.

This project is still in development and we focus our efforts in developing a system to the production and accumulation of fatty acids in these structures. More testing must be done to determine the exact composition (nutrient-like) of these structures, but it is possible that they can also be a source of vitamins (α-tocopherols, ascorbic acid, β-carotene, and glutathione), anti-oxidants (betalains); and minerals since these compounds are accumulated naturally in the plant tissue that originated these structures.

Some commercial interest has been shown in the use these structures as a supplement in animal feed, in order to increase essential fatty acid content in the animal tissues and by-products that indivertible will be used in human diet.

9. Future Outlook

Purslane is an important plant system with great potential not only as a food source, but also as a scientific source of information about plant-environment relations. Its high tolerance to drought and salinity stresses together with its high nutritional value makes this plant a valuable food crop for the future. The work developed in this thesis revealed a possible regulation of *P. oleracea* desaturase genes in response to changes in the plant environment, particularly *FAD8* gene. In future work it would be interesting to study in more detail the regulatory mechanisms associated to this gene and the role the overexpression of this gene in the plant tolerance to environmental stresses such as salinity and low temperatures. Heterologous expression of this gene in yeast system or tobacco cell culture could be also used as a tool to evaluate the ability of this gene to confer tolerance to environmental stresses to other plant species. Another possible approach, already used with *Arabidopsis thaliana*, is the construction of *P. oleracea* mutant's lines deficient in one or more desaturase genes. These lines would be a useful tool to understand the role of desaturase genes in the plant survival and tolerance to environmental stresses.

Another project we develop during the completion of this thesis was the development of *P. oleracea* cell cultures lines and optimization of a system to produce polyunsaturated fatty acids in liquid cell culture. Due to the elevated resistance, proliferative *callus* growth, and regeneration capacity of *P. oleracea* in cell culture conditions, it is possible that this plant can be used as a competitive culture system for heterologous expression of interest's genes, as tobacco. The results generate in this work were the basis for a patent application that is under evaluation now. In the near future we aim to optimize culture conditions in order to decrease production cost and/or increase products yield in order to obtain a commercially viable method able to compete with the established systems. Another goal is to explore this system for the production of other compounds of interests, such as betalains (antioxidant properties), that naturally are produced in *P. oleracea* tissues.

10. Acknowledgments

This thesis is the completion of a cycle that started many years ago, at the Department of Food Engineering, in the University of Algarve, where I completed the Licentiate in Food Engineering and met my PhD supervisor Isabel S. Carvalho. Further on I was invited by her to follow an interesting project that started as my master studies in plant biochemistry and evolved to become the base of my PhD studies. Along the way contacts were made and I end up doing a significant part of my PhD in the Faculty of Applied and Natural Sciences, in Kalmar University, in Sweden, under the supervision of Maria Brodelius, whom I thank for the opportunity of working with extraordinary people that helped me along the way. It was a hard path, but I had help from many people and organizations that made the production of this thesis possible.

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12. Appendices

Appendice 1 - Paper I: Monica C. Teixeira and Isabel S. Carvalho (2008). Effects of salt stress on purslane (*Portulaca oleracea*) nutrition. Annals of Applied Biology, 154 (1), 77-86.

Appendice 2 - Paper II: Molecular Cloning and expression analysis of three omega-6 desaturase genes from purslane (*Portulaca oleracea* L.) (In press, Biotechnology Letters)

Appendice 3 - Paper III: Omega-3 fatty acid desaturase genes isolated from purslane (*Portulaca oleracea* L): Expression in different tissues and response to cold and wound stress. (Submitted: article ID: jf-2009-009015)

Appendice 4 - Paper IV: Differential expression of omega-3 and omega-6 desaturase genes and linolenic/linoleic accumulation levels in leaves of *Portulaca oleracea* L., exposed to chloride-salinity treatments. (Submitted: article ID: Planta-2009-03-0151)

Appendice 5 - Paper V: Establishment of in vitro plants, cell and tissue cultures from *Portulaca oleracea* for the production of polyunsaturated fatty acids (Manuscript)

PAPER I

Monica C. Teixeira and Isabel S. Carvalho (2008). Effects of salt stress on purslane (*Portulaca oleracea*) nutrition. Annals of Applied Biology, 154 (1), 77-86.

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RESEARCH ARTICLE

Effects of salt stress on purslane (*Portulaca oleracea*) nutrition

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Abstract

The objective of this study was to determine the influence of saline stress on the chemical composition of purslane (*Portulaca oleracea*), in particular the mineral composition. Four salinity levels were investigated using irrigation solutions with electrical conductivity values of 0.8, 6.8, 12.8 and 24.2 dS m^{-1} and two planting dates (May and July) were tested. Samples of full-grown leaf and stems of purslane were harvested after 7 and 15 days of the saline treatment exposure. Chemical analysis (dry matter basis) of leaves showed significant differences among the different saline treatments for all the characteristics measured. Salinity levels, planting date and harvest time significantly influenced ($P < 0.05$) the levels of crude protein, total lipids, ash and carbohydrate content. Salinity treatments did not significantly ($P > 0.05$) affect the water content of purslane leaves. The crude protein content of purslane leaves decreased with increasing salinity levels and time of exposure to treatment. However, carbohydrates and mineral residue content increased. An unusual phenomenon was noted for intermediate salinity levels, whereby an increase in total lipid content was measured in leaves of plants exposed to salinity treatments of 6.8 and 12.8 dS m^{-1}. The highest mineral residue content was seen in leaves of purslane exposed to the highest salinity treatment. The mineral composition was also affected by salinity levels, Na and Cl uptake, and accumulation increased with increasing salinity in irrigation solution; Mg concentration was not significantly ($P > 0.05$) affected by salinity levels, although a slight increase was seen, and Ca, K and Zn levels significantly ($P < 0.05$) decreased. Ca and Zn preferentially accumulated in the leaves, while K and Na values were higher in the stems. A significant increase ($P < 0.05$) in relative ratio of Na/K, Mg/K, Na/Ca and Mg/Ca was observed with increasing salinity levels. A decrease in the yield of purslane was only observed for the most severe saline treatment, where the highest ratio of Mg/Ca was seen. This study reveals that purslane is relatively tolerant to conditions of moderate salinity, thus improving its potential to become a key vegetable crop for animal and human consumption.

Introduction

Plants need essential mineral elements for their growth and development, but the presence of soluble salts in excess in the soil is harmful to the majority of plants. In fact, the most limiting factor on plant growth, worldwide, is soil salinity (Singh & Chatrath, 2001; Zhu, 2003). Soil salinity levels in the Mediterranean coast, in 1997, ranged from 0.1 to 0.2 dS m^{-1}. In the same period of time, the water table decreased by 0.1–0.5 m. These conditions resulted from sea water intrusion and the arid climate of this region (Aksoy *et al.*, 2003). Salinity is an important problem in arid and semi-arid regions. The major commodities threatened by salinity are agricultural crops (Vasconcelos, 1987; Aksoy *et al.*, 2003). The Algarve has high water requirements because of increased demands from tourism during the summer months and the increase in golf course construction (Vasconcelos, 1987). The main factors responsible for soil salination are the low quality of irrigation waters, excessive fertilisation and the deficient drainage of some soils. As a result the yield of crops decreases and it is no longer possible to grow more salt-sensitive species (Vasconcelos, 1987; Grieve, 2000; Singh & Chatrath, 2001). An approach to the salinity problem is the use of crops with a higher tolerance to soil salinity (Neumann, 1997; Shannon *et al.*, 2000), although all plants are affected by salinity leading to general inhibition of growth (Neumann, 1997; Grieve, 2000). Salt tolerance can be adequately measured on the basis of two parameters: the threshold (EC_t), the electrical conductivity (ECi) that is expected to cause the initial significant reduction in the maximum expected yield (Y_{max}) and the slope (s). The slope is simply the percentage of yield expected to be reduced for each unit of added salinity above the threshold value. The 'weed' purslane (*Portulaca oleracea* L.) deserves special attention by agriculturalists and nutritionists alike. Purslane is a very versatile plant; it colonises all kinds of soils (Matthews *et al.*, 1993; Kamil *et al.*, 2000). Purslane has been rated as moderately tolerant with a salinity threshold of 6.3 dS m^{-1} (EC_t) and a slope of 9.6% (Maas & Grattan, 1977; Kumamoto *et al.*, 1990). However, after the first cutting, the halophytic nature of purslane is expressed and the salt tolerance of purslane increases with subsequent harvests (Grieve & Suarez, 1997). Purslane has been studied in detail as a prolific weed, but very little is known about its production as a food crop and the effects of cultural conditions on its nutritional value, although there have been some studies carried out to determine the best cultural conditions to obtain higher levels of fatty acids (Palaniswamy *et al.*, 2001) and lower levels of oxalic acid (Palaniswamy *et al.*, 2002) in purslane leaves, under greenhouse conditions. Many of the pharmacological properties attributed to purslane in folk medicine may, in fact, be based on the efficacy of some of its constituents, which have now been identified by modern analytical methods. The shoot is a rich source of ω-3 fatty acids (Simopoulos & Salem, 1986; Kumamoto *et al.*, 1990; Omara-alwala *et al.*, 1991; Palaniswamy *et al.*, 2001), α-tocopherols, ascorbic acid, β-carotene and glutathione (Simopoulos *et al.*, 1992), and it is also rich in various minerals with large amounts of potassium and magnesium (Mohamed & Hussein, 1994). The seeds contain 21% protein and 20% oil, of which the major constituents are linoleic (46%) and linolenic (31%) acids. The nutritional properties of purslane (Simopoulos *et al.*, 1995; Palaniswamy *et al.*, 2002; Simopoulos, 2004) and its tolerance to saline environment make this plant a promising candidate for irrigation with residual waters (Kumamoto *et al.*, 1990; Grieve & Suarez 1997). The objective of this study was to determine the influence of saline stress on the chemical composition of purslane cultures, in particular the mineral composition.

Materials and methods

Plant culture and flour preparation

Golden leaf purslane seeds, obtained from local markets, were sown in trays filled with soil/vermiculite mixture (70:30) in spring (March) and summer (July) of 2004. Five-week-old seedlings were transplanted into pots (500 cm^3) filled with the same soil mixture and placed in a glasshouse. Each pot contained four seedlings. Plants were irrigated once a day (100 mL) using an irrigation solution with a mean composition (mg L^{-1}) of 2.3 K^+, 59 Na^+, 97 Ca^{2+}, 38 Mg^{2+}, 28 SO_4^{2-}, 6 HCO_3^- and 110 Cl^-, with an average ECi value of 0.8 dS m^{-1}. This solution was used as the control treatment. No nutritive supplements were added in any of the irrigation solutions during the experiment. The volume of irrigation solution allowed the soil mixture to be completely saturated. Seven days after the seedlings were transplanted, three saline treatments were imposed with irrigation solutions, so that the ECi mean values for the four treatments were: 0.8 (control), 6.8, 12.8 and 24.2 dS m^{-1}, which corresponded to 0, 60, 120 and 240 mM NaCl, respectively. Pots were arranged in a randomised block design with three blocks (replicates) of treatment combinations (season by concentration by time). Air temperatures in the glasshouse ranged from 5°C to 25°C (mean = 20°C) in the spring test and from 25°C to 40°C (mean = 30°C) in the summer experiment. Relative humidity ranged from 16% to 87% in both seasons. On 7 and 15 days after the completion of salination (hereafter designated as harvest 1 and 2), three plants were cut at soil level and weighed. Each plant was picked at random from each pot and in each harvest from the same pot. The culture yield was calculated based on fresh weight of the plants, according to Kumamoto *et al.* (1990).

Plant tissue analysis

The individual samples of plant material were separated into leaves and stems. Chemical composition was determined only on leaves, and mineral content was determined in both leaves and stems. All plant materials were dried in a forced-air oven at 60°C for 96 h and ground to a powder in a laboratory scale hammer mill (Tecator, AB, Hogana, Sweden Cyclotec 1093) set up with a 1-mm opening screen. The resultant flour was placed in airtight glass jars and stored at 20°C until analysis. The flour from each sample was analysed for ash, moisture, crude proteins and oil using the Association of Official Analytical Chemist (AOAC, 1990) methods, according to procedure numbers: 7.010, 14.114, 2.049, 7.054 and 14.08, respectively. Percentage total carbohydrate content in purslane leaves was determined by subtraction of the percentual values of the other nutrients, protein, lipids and ash fractions and also includes soluble sugars and fibres. Results are the mean of triplicate determinations and expressed as percentage on a dry matter basis.

Mineral composition

Ashing was carried out by means of incineration in a muffle oven at 450 ± 25°C until an ash was obtained. The ash was acid digested (HCl) for the determination of micro and macro elements. Potassium (K) and sodium (Na) were measured by a flame absorption spectrophotometer (model AA 1475; Varian, NJ, USA). Intra-test, using 10 samples of a solution, with known concentrations of K and Na was performed, with coefficients of variation of 3.4% and 2.0% for K and Na, respectively. The elements iron (Fe), calcium (Ca), magnesium (Mg) and zinc (Zn) were measured by an atomic absorption spectrophotometer (Perkin Elmer model 3100, Norwalk, CT, USA) equipped with hollow cathode lamps. Recovery of added known amounts of standards to samples gave 98–105% of expected values for all minerals. Chloride (Cl) was determined by direct potentiometry, with a chloride-selective electrode, according to Rieger & Litvin (1998). Results are the mean of triplicate determinations and expressed as mmol kg^{-1} dry matter.

Statistical analysis

Plant yield, proximal nutritional composition and mineral content were subjected to an analysis of variance (ANOVA) using the general linear model procedure of the SAS Statistical Software. For the analysis, data were combined from the two sowing seasons (spring and summer) because the same cultivation conditions and all other protocols were applied. Differences in time of harvest and salinity treatments were determined with ANOVA using a split-plot model with replicates as the blocks, pots as the main plot factor and time of harvest as the subplot factor. No transformation to data was required for any of the ANOVAs. Significant differences between means were determined by least significant difference, with a significance level of 0.05.

Results and discussion

Culture yield

Moons *et al.* (1995) reported on varietal difference in the growth of roots and leaves, for a salt-sensitive and two salt-resistant varieties of rice, within 10 days of root exposure to 50 mol m^{-3} NaCl. Importantly, no other visible symptoms of stress (e.g. salt-accelerated leaf senescence) were observed, that is osmotic responses could have been involved. Purslane plants also did not exhibit any visual symptoms of sodium chloride toxicity or nutrient deficiency during the 15 days of saline treatments. Salinity-induced reduction in plant tissue production compared with control was only seen in plants exposed to irrigation solution with ECi greater than 6.8 dS m^{-1} in both experiments. In plants exposed to irrigation solutions with ECi value of 24.2 dS m^{-1}, the yield reduction ranged from 29% to 32% and from 29% to 44% in the summer and spring tests, respectively. This response to increased salinity in the early harvest is similar to the yield response reported by Kumamoto *et al.* (1990) for chloride salinity but with a lesser decrease in yield. Other plants have shown a decrease in culture yield when exposed to saline environments. D'Amico *et al.* (2003) reported a marked reduction in the yield of tomatoes cultures (*Lycopersicon esculentum* Mill.) exposed to a saline environment, which at 8 and 14 dS m^{-1} ECi, respectively, were 69% and 43% of the control (3 dS m^{-1}). Lacerda *et al.* (2003) also reported a reduction in the yield of two forage sorghum genotypes (*Sorghum bicolor* L.) differing in salt tolerance when exposed to salinity levels of 100 mM NaCl.

Proximal nutritional composition

The proximal nutritional composition of purslane leaves was significantly ($P < 0.05$) affected by the salinity level of the treatment, time of harvest and season of the year when the test was conducted (Table 1), and also the three-way interaction of season, salinity and harvest time was significant ($P < 0.05$) for all measurements. A higher content of nutrients was observed in the test conducted in the summer, which may be related to the fact that purslane is a semi-tropical plant (Vengris *et al.*, 1972; Matthews *et al.*, 1993), and higher temperatures favoured the growth and nutrient accumulation in the tissues.

Succulence is an important property of halophytic or saline-tolerant plants because a high number of halophytic

Table 1 Average proximal nutritional composition (% DW) of leaves collected from cultures of purslane (*Portulaca oleracea* L.) exposed to four levels of salinity: ECi = 0.8 (control), 6.8, 12.8 and 24.2 dS m^{-1}, considering two sowing seasons, spring and summer[a]

			Nutrient Content (% DW)				
Season	Harvest (days)	ECi (dS m^{-1})	Total Protein	Total Lipids	Ash	Total Carbohydrates[b]	Water Content
Spring	7	0.8	15.53	7.28	11.11	66.08	91.21
		6.8	13.48	14.96	12.14	59.42	90.80
		12.8	10.71	10.16	12.91	66.22	89.96
		24.2	10.59	4.71	13.38	71.32	90.58
	15	0.8	17.47	8.79	12.23	61.51	91.85
		6.8	13.69	13.73	15.32	57.25	91.58
		12.8	11.66	6.56	20.54	61.24	91.87
		24.2	10.67	4.64	24.36	63.33	90.09
Summer	7	0.8	19.07	9.20	14.08	57.65	92.54
		6.8	17.47	20.15	14.71	47.66	92.39
		12.8	15.79	17.05	14.78	52.38	92.24
		24.2	12.19	6.85	14.06	66.55	91.61
	15	0.8	15.54	8.68	14.04	61.74	90.67
		6.8	13.09	13.75	16.63	56.53	90.74
		12.8	10.20	7.75	20.74	61.31	90.15
		24.2	8.46	7.19	20.80	66.51	89.92
LSD[c] (DF = 16)			0.15	0.20	0.38	0.45	0.07
LSD[d] (DF = 14)			0.10	0.14	0.27	0.32	0.04

DF, degree of freedom; DW, dry weight; ECi, electrical conductivity; LSD, least significant difference.

[a] Separation of means was based on Student's *t*-test ($P = 0.05$, $n = 48$).

[b] Values determined by total nutrient content (% DW) less protein, lipids and ash.

[c] LSD (Student's *t*-test) between means ($P < 0.05$); comparison for any pair of means across harvest time, with the same season and NaCl concentration.

[d] LSD (Student's *t*-test) between means ($P < 0.05$); all other comparisons.

plants show some kind of succulence in their tissues. Ottow *et al.* (2005) reported the development of leaf succulence and osmotic adjustments in the salt-tolerant tree *Populus euphratica* exposed to sodium chloride salinity, from 25 to 400 mM NaCl. Cytoplasmic toxicity is rapidly reached when the central vacuole of the cell becomes saturated with ions, so that the plants with large vacuoles can store more ions without reaching toxic levels in the cytoplasm (Nilsen & Orcutt, 2000). The C4 dicotyledonous *P. oleracea* possesses succulent leaves with branched venation and is composed of various types of cells. One of these is water storage cells that are particularly large, vacuolated and contain few small chloroplasts (Lara *et al.*, 2003). In this study, mean water content of purslane leaves slightly decreased when the salinity level of the treatment was raised. In the spring, after 7 days of treatment (harvest 1), there was a reduction in mean water content with increasing salinity; however, this decrease was only a significant change ($P < 0.05$) to the highest level, 24.2 dS m^{-1} after 15 days of treatment (harvest 2) (Table 1). In the summer, a similar result was obtained, with a decrease in mean water content in the highest level of salinity treatment, 24.2 dS m^{-1}, after 7 and 15 days of treatment (harvests 1 and 2) and also for the intermediate level of treatment, 12.8 dS m^{-1} after 15 days of treatment (harvest 2). These data suggest that, although the plants were exposed to saline stress, they were able to maintain the water levels in the leaves. The time of exposure of the plants to the saline treatment did not affect the water level of purslane leaves in the spring test, but in the summer experiment, the moisture content was around 2% lower after 15 days of saline treatment. Higher temperatures may have led to higher levels of transpiration in purslane leaves, which might explain the decrease in the total water content in the plants exposed to longer treatments in the summer test.

Many studies refer that an increase in salinity levels in a plant's surrounding environment leads to a decrease of nitrogen uptake and accumulation by the root (Neumann, 1997; Nilsen & Orcutt, 2000). In many plants, protein synthesis is affected by the exposure of the plant to sodium chloride, and in some cases, protein hydrolysis occurs with the release and accumulation of free amino acids in the tissues (Levit, 1980; Neumann, 1997; Nilsen & Orcutt, 2000). Mean total protein in purslane leaves ranged from 10.59% to 17.47% of total protein in the spring test and from 8.46% to 19.07% total protein in the summer test (Table 1). The protein levels in purslane cultures (control plants) were similar to or higher than those of other forage or vegetable food crops traditionally used as protein sources for humans and animals.

These high crude protein values were also reported by Ezekwe *et al.* (1999) and Obied *et al.* (2003) and placed purslane above alfalfa, which, with a crude protein content of 17% DW, is currently the most important commercial vegetable crop in the USA. The highest mean total protein content was present in control plants and the lowest in plants exposed to the extreme saline treatment, 24.2 dS m^{-1}, in both seasons. Mean total protein level was higher and significantly different ($P < 0.05$) from the summer experiment. The effects of salinity stress in protein synthesis were also more evident in the summer, revealed by a substantial decrease in the leaves' protein content, when salinity levels in the irrigation solution increased. The length of treatment exposure significantly affected ($P < 0.05$) the average total protein content of purslane leaves, which was lower after 15 days of saline treatment (harvest 2) in the summer assay (Table 1). In the spring assay, the mean total protein value was higher after 15 days of exposure to treatment, at all salinity levels, except for the 24.2 dS m^{-1} treatment where no change was observed (Table 1). Many of the protein synthesis steps are very sensitive to alterations in the ionic equilibrium in the cells, which may result in a complete blockage of protein metabolism (Levit, 1980; Neumann, 1997; Singh & Chatrath, 2001). These data suggest that, although the protein biosynthesis was adversely affected by the salinity, the plant ability to synthesise protein was not totally compromised by the salinity levels used. Furthermore, the increase in uptake and accumulation of chloride ions (Cl^-) in plant tissue generally results in the decrease in nitrate (NO_3^-) accumulation in the plant's aerial parts (Rieger & Litvin, 1998; Nilsen & Orcutt, 2000; Ottow *et al.*, 2005). Examples of this have been found in cucumber, *Cucumis sativus* L. (Martinez & Cerdá, 1989), eggplant, *Solanum melongena* L. (Savvas & Lenz, 1996), melon, *Cucumis melo* L. (Feigin *et al.*, 1987; Kafkafi *et al.*, 1992), tomato, *Lycopersicon esculentum* (Kafkafi *et al.*, 1982, 1992; Feigin *et al.*, 1987; Pérez-Alfocea *et al.*, 1993) and barley, *Hordeum vulgare* L. (Britto *et al.*, 2004). Many have attributed this reduction to Cl^- antagonism of NO_3^- uptake (Feigin *et al.*, 1987; Savvas & Lenz, 1996). In this study, lower amounts of mean total protein were found in purslane leaves in the spring experiment, where higher levels of chloride accumulation were observed (Table 1).

It has been reported that *P. oleracea* is the richest source of total lipids as well as soluble carbohydrates in edible leafy vegetables surveyed (Kesden & Will, 1987; Xiang *et al.*, 2005). Mean total lipid content in purslane leaves ranged from 4.64% to 14.96% DW in the spring test and from 6.85% to 20.15% DW in the summer test. Mean total lipid content in purslane leaves from control cultures ranged from 7.28% to 9.20% DW, with an average value of 8.24% DW (Table 1). Such high lipid content was also reported by Ezekwe *et al.* (1999) and Obied *et al.* (2003). In the spring test, mean total lipid content in purslane leaves of plants exposed to 6.8 and 12.8 dS m^{-1} was 2.0-fold and 1.4-fold higher than control plants after 7 days of treatment, but after 15 days, only the plants exposed to 6.8 dS m^{-1} had a value higher than the control plants, with a 1.6-fold increase (Table 1). A similar result was obtained in the summer test, with a 2.2-fold and 1.9-fold increase for plants exposed to 6.8 and 12.8 dS m^{-1} after 7 days and a 1.6-fold increase in the mean total protein content of plants exposed to 6.8 dS m^{-1} after 15 days (Table 1). In both cases, the mean total lipid value in plants exposed to 12.8 dS m^{-1} after 15 days is lower than the control plants. Heuer *et al.* (2002) reported an increase in the total lipid content of *Oenothera biennis* seeds when the plant was exposed to moderate saline environments (ECi $\leq$6 dS m^{-1}). These data suggest that moderate levels of salinity (ECi $\leq$12.8 dS m^{-1}) may lead to an increase in lipid synthesis in plant tissue, but at higher levels of salinity, the lipid synthesis was compromised (Table 1).

Mean total ash content in purslane leaves ranged from 11.11% to 24.36% DW in the spring experiment and from 14.08% to 20.80% DW in the summer experiment. Mean total ash content of control plants in both experiments (Table 1) were less than those measured by Ezekwe *et al.* (1999) and Mohamed & Hussein (1994). The total ash content of purslane leaves increased with the salinity levels in the irrigation solutions (Table 1). This fact may be related to the increase in uptake and accumulation of sodium and chloride ions (Table 2) in plants subjected to saline treatments. An increase in total ash content in the halophyte plant *Atriplex griffithii* var. *stocksii* shoots was reported by Khan *et al.* (2000); the increase was also more significant in the leaves.

The accumulation of organic compounds, such as sugars and amino acids, in the cytoplasm plays an important role in the osmotic balance of plants (Morgan, 1992). Mean total carbohydrate content in purslane leaves was determined in relation to the other nutrients, by subtracting the values (in percentage) of total protein, total lipid and ash contents and included soluble sugars and fibres. In the control plants, the total carbohydrate content ranged from 58% to 66% (Table 1), which was higher than that reported by Obied *et al.* (2003). Higher amounts of total carbohydrates in purslane leaves were observed in plants exposed to the highest salinity level, 24.2 dS m^{-1}, compared with control samples in both experiments, at both harvests ($P < 0.05$). A slight increase in total carbohydrates was also observed in plants exposed to 12.8 dS m^{-1} but was not significantly ($P > 0.05$) different from control plants. For moderate salinity conditions,

Table 2 Average concentrations, on a DW basis, of sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn) and chloride (Cl) in leaves collected from cultures of purslane (*Portulaca oleracea* L.) exposed to four levels of salinity: ECi = 0.8 (control), 6.8, 12.8 and 24.2 dS m^{-1}, considering two sowing seasons, spring and summer[a]

Season	Harvest (days)	Treatment ECi (dS m^{-1})	Mineral Concentration (mmol kg^{-1} DW)						
			Ca	Mg	Na	K	Fe	Zn	Cl
Spring	7	0.8	1665	2009	377	1146	195	128	82
		6.8	1325	1949	558	1089	139	103	171
		12.8	1075	2021	744	1060	133	90	185
		24.2	938	2008	705	997	109	88	256
	15	0.8	2180	2052	458	1182	195	144	158
		6.8	1617	2096	787	1031	171	130	294
		12.8	1541	1992	148	947	112	114	519
		24.2	1150	1945	1936	773	109	109	804
Summer	7	0.8	847	1815	421	1032	122	132	123
		6.8	924	1668	480	1019	136	97	160
		12.8	964	1957	698	918	80	92	212
		24.2	690	2031	1019	867	133	66	257
	15	0.8	813	1770	618	1173	151	135	151
		6.8	791	2103	864	1163	160	117	159
		12.8	759	2053	1182	1078	150	50	214
		24.2	445	2070	1608	746	288	33	394
LSD[b] (DF = 16)			16.8	31.4	7.7	67.8	8.7	1.5	14.5
LSD[c] (DF = 14)			12.0	22.4	5.5	48.4	6.2	1.1	10.3

DF, degree of freedom; DW, dry weight; ECi, electrical conductivity; LSD, least significant difference.

[a] Analysis was applied to the combined data from the two sowing seasons, two harvest times and four levels of chloride salinity in the irrigation water. Separation of means was based on Student's *t*-test ($P = 0.05$, $n = 48$).

[b] LSD (Student's *t*-test) between means ($P < 0.05$); comparison for any pair of means across harvest time, with the same season and NaCl concentration.

[c] LSD (Student's *t*-test) between means ($P < 0.05$); all other comparisons.

6.8 dS m^{-1}, a decrease in total carbohydrate content was observed relative to control plants (Table 1). An increase in carbohydrates content because of saline treatment has also been measured in other plants. Kafi *et al.* (2003) reported an increase in total carbohydrate content in leaves of wheat cultivars with increasing salt content in the medium (NaCl ≤300 mM). Watanabe *et al.* (2000) reported an increase in the total sugar content of young leaves of *Po. euphratica* for NaCl concentration of 250 mM, in an *in vitro* test.

Mineral composition

Ions can interact with the soil and the plant in different ways, which can lead to deficiency or toxicity phenomena that affect growth and development (Nilsen & Orcutt, 2000; Zhu, 2003). The ionic uptake by the cell is affected by the environmental salinity, which affects the relative availability of the ions in the area surrounding the root (Grattan & Grieve, 1999; Nilsen & Orcutt, 2000). The mineral composition of purslane leaves and stems were significantly ($P < 0.05$) affected by the salinity level of the treatment, time of harvest and season of the year when the test was conducted, and also the three-way interaction of season, salinity and harvest time was significant ($P < 0.05$) for all measurements. In this study, salinity significantly affected the ion concentration and distribution within the purslane leaves (Table 2) and stems (Table 3). In general, the uptake and accumulation of Na and Cl ions in purslane tissues were not significantly affected ($P > 0.05$) by the season when the experiment took place, although a substantial increase in the stem Cl was observed in the summer, with mean level significantly different compared with the spring ($P < 0.05$). In the spring experiment, when the lowest temperatures were measured, the ion uptake and accumulation in the leaves of purslane plants was lower, with the exception of Ca and K, with higher values in this season. The same was observed for Fe and Zn ion accumulation in stems, but some variation in Ca, Mg and K ion accumulation was observed. Stem Ca and Mg mean concentrations were lower in the spring test after 7 days of treatment; however, after 15 days, the accumulation of these ions in the spring assay was higher than that observed in the summer assay. Stem K values were higher in the spring assay after 7 days of treatment and lower after 15 days compared with the summer assay. This variation may be related to the ion movement and preferential accumulation in the plant tissues. With increasing salinity, the plants need more Ca.

Table 3 Average concentrations, on a DW basis, of sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn) and chloride (Cl) in stems collected from cultures of purslane (*Portulaca oleracea* L.) exposed to four levels of salinity: ECi = 0.8 (control), 6.8, 12.8 and 24.2 dS m^{-1}, considering two sowing seasons, spring and summer[a]

			Mineral Concentration (mmol kg^{-1} DW)						
Season	Harvest (days)	Treatment ECi (dS m^{-1})	Ca	Mg	Na	K	Fe	Zn	Cl
Spring	7	0.8	503	1792	509	2282	222	74	92
		6.8	355	1724	727	2153	165	63	121
		12.8	310	1727	728	1907	137	67	153
		24.2	258	1941	960	1872	124	36	161
	15	0.8	730	1784	496	1611	52	66	176
		6.8	510	1778	949	1558	64	54	241
		12.8	456	1750	1919	1550	126	56	475
		24.2	403	1723	2370	1482	123	59	541
Summer	7	0.8	951	2036	452	1711	136	199	616
		6.8	906	2061	624	1671	102	122	1341
		12.8	447	2063	869	1602	128	52	2097
		24.2	339	1947	1188	1528	132	40	2149
	15	0.8	536	2036	625	1897	175	99	471
		6.8	398	2018	975	1790	132	83	1000
		12.8	349	2099	1293	1647	178	82	1440
		24.2	201	2036	2010	1105	231	40	2338
LSD[b] (DF = 16)			10.4	36.4	11.1	11.0	3.0	7.3	29.6
LSD[c] (DF = 14)			7.4	26.0	7.9	7.8	2.1	5.2	21.1

DF, degree of freedom; DW, dry weight; ECi, electrical conductivity; LSD, least significant difference.

[a] Analysis was applied to the combined data from the two sowing seasons, two harvest times and four levels of chloride salinity in the irrigation water. Separation of means was based on Student's *t*-test ($P = 0.05$, $n = 48$).

[b] LSD (Student's *t*-test) between means ($P < 0.05$); comparison for any pair of means across harvest time, with the same season and NaCl concentration.

[c] LSD (Student's *t*-test) between means ($P < 0.05$); all other comparisons.

Simultaneously, the transport of this ion can be reduced by ionic interactions, precipitation and the higher ionic strength of the medium, which leads to a reduction of the Ca availability for the plant (Grattan & Grieve, 1999; Nilsen & Orcutt, 2000). When Ca and Mg concentrations in the medium fall below the critical limits of the plant cell, the K uptake also decreases (Grattan & Grieve, 1999). In this study, the increase of NaCl concentration in the irrigation solution resulted in a decrease in the accumulation of Zn, Ca and K in plant tissues, as well as an increase in Na and Cl concentrations.

A slight increase in the Mg concentration was observed in leaves and stems of purslane plants exposed to higher levels of saline treatment, 12.8 and 24.2 dS m^{-1}, in both seasons (Tables 2 and 3). The effect of the saline treatments on Fe accumulation was not clear. In the spring test, leaf Fe concentration decreased with the increase of salinity in the irrigation water. Stem Fe also decreased with increasing salinity after 7 days of treatment exposure, but after 15 days, the stem Fe values were higher for the plants exposed to higher levels of saline treatment. In the summer test, exposure to treatments of 6.8 and 24.2 dS m^{-1} leads to a higher leaf Fe concentration in relation to control plants. Stem Fe concentration from plants exposed to 12.8 dS m^{-1} treatment did not differ significantly ($P > 0.05$) in relation to control plants, and the highest stem Fe value was obtained for the 24.2 dS m^{-1} treatment after 15 days of exposure. The lowest stem Fe concentrations were obtained for plants exposed to 6.8 dS m^{-1} treatment.

The environmental salinity affects not only the nutrient uptake but also the distribution and accumulation in the plant tissues. In a medium where the Na ions are at a higher concentration, a reduction on the uptake and mobility of calcium ions to the growing areas will affect the vegetative and reproductive organs of the plant (Grattan & Grieve, 1999; Singh *et al.*, 2001). Khan *et al.* (2000) measured an increase in Na and Cl accumulation in shoots of *A. griffithii* var. *stocksii*, together with a decrease in Ca, K and Mg, this effect being more evident in the leaves. In this study, we observed a differential accumulation of the Na, K, Ca and Mg in plant organs as seen by Grieve & Suarez (1997), with a preferential accumulation of Ca in the leaves and Na and K in the stems. Apparently, the increase in Na uptake and accumulation led to a reduction of Ca accumulation in the purslane leaves and stems. At all salinity levels, stem Ca was roughly 30% of that in the leaves (Tables 2 and 3) and decreased with the increase in salinity in the irrigation solution. Although a preferential accumulation of Mg in purslane leaves, previously seen by Grieve & Suarez (2000), was not observed, this ion was present in significant amounts in purslane tissues. Ca and K deficiencies induced by the increase in salinity may be responsible for the reduction in plant growth and yield seen in the highest saline treatment, 24.2 dS m^{-1}.

High concentrations of sodium and chloride ions in soil or irrigation solutions may reduce the uptake and accumulation of ions and nutrients, leading to extreme ratios of Na/Ca, Na/K, Ca/Mg and Cl/NO_3 (Grattan & Grieve, 1999). In high salinity conditions, high concentration of Na not only interferes in K uptake at root level but also causes alterations in the integrity and selectivity of membranes. Studies performed in a large variety of vegetables revealed a significant reduction of K levels in the plant tissues with the increase in Na and/or increase in Na/Ca ratio in the roots (Pérez-Alfocea *et al.*, 1993; Gorham, 1994; Grattan & Grieve, 1999). As salinity increased, purslane leaves and stems Ca and K concentrations decreased, while Na and Mg concentrations increased (Tables 2 and 3) in both seasons, although the decrease was more evident in the summer. To verify some ionic interactions, Na/Ca, Mg/Ca, Na/K and Mg/K ratios were calculated for plants exposed to 15 days of saline treatment (harvest 2), based on individual determinations of each ion, using corresponding mean values in Tables 2 and 3. All ionic ratios increased as a result of the increase in NaCl concentration in the irrigation solution and the exposure time. This increase was more clearly observed in the leaves and stems of plants exposed to the more severe treatments (Table 4). Gorham (1994) reported that at lower external salinities (<100 mM NaCl), the enhanced K/Na discrimination character of wheat cultivars resulted in a low leaf Na concentration and the maintenance of K concentration similar to those found in plants not grown in the presence of salt. K and Na concentrations were relatively unaffected for the tested salinity levels. Data from the experiment on purslane (Tables 2 and 3) would therefore seem to suggest that this character may also be present in this species because the K concentrations in leaves of plants exposed to the lower (6.8 dS m^{-1}) and intermediate (12.8 dS m^{-1}) salinity treatments were not much lower than the values seen in control plants. Both Na/K and Mg/K in leaves and stems increased as the K and Mg concentrations remained constant while salinity increased (Table 4). In the spring test and for the highest level of salinity, leaf Na/K and Mg/K ratios increased 6.5-fold and 1.5-fold, respectively, in relation to controls; stem Na/K ratio increased in the order of fivefold and there was only marginal increase in Mg/K ratio. In the summer experiment, leaf Na/K and Mg/K ratios increased fourfold and twofold, respectively, and stem Na/K and Mg/K ratios increased fivefold and twofold, respectively (Table 4). Mg/Ca ratios, higher than one, led to a reduction of the growth and yield of cereals, such as wheat and sesame (Gorham, 1994; Yahya, 1998). In this study, Mg/Ca ratios were always greater than unity, at all salinity levels, both in the leaves and stems, but a reduction of the growth and yield of purslane cultures was only seen in the highest level of salinity.

Conclusions

The genus *Portulaca*, with more than 100 species, is found in every region of the world. *P. oleracea* has the potential for becoming a highly nutritional vegetable for both animal (fodder) and human consumption. Salinity affected the growth and yield of purslane plants, although a yield reduction was only observed in plants exposed to the highest level of salinity. Salinity also significantly affected the proximal nutritional composition of purslane leaves with a decrease in potential

Table 4 Ion selectivity ratios in purslane leaves and stems[a]

Plant Tissue	Treatment ECi (dS m^{-1})	Spring				Summer			
		Na/Ca[b]	Na/K[b]	Mg/Ca[b]	Mg/K[b]	Na/Ca[b]	Na/K[b]	Mg/Ca[b]	Mg/K[b]
Leaves	0.8	0.2 (0.01)	0.4 (0.01)	0.9 (0.02)	1.7 (0.01)	0.8 (0.07)	0.5 (0.01)	2.2 (0.18)	1.5 (0.03)
	6.8	0.5 (0.04)	0.8 (0.01)	1.3 (0.11)	2.0 (0.04)	1.1 (0.02)	0.7 (0.01)	2.7 (0.01)	1.8 (0.07)
	12.8	1.0 (0.05)	1.6 (0.13)	1.3 (0.02)	2.1 (0.02)	1.6 (0.01)	1.1 (0.01)	2.7 (0.09)	1.9 (0.07)
	24.2	1.7 (0.03)	2.5 (0.10)	1.7 (0.04)	2.5 (0.12)	3.6 (0.03)	2.2 (0.02)	4.7 (0.12)	2.8 (0.08)
Stems	0.8	0.7 (0.01)	0.3 (0.01)	2.4 (0.06)	1.1 (0.01)	1.2 (0.08)	0.3 (0.06)	3.8 (0.06)	1.1 (0.04)
	6.8	1.7 (0.05)	0.6 (0.01)	3.5 (0.10)	1.1 (0.03)	2.5 (0.06)	0.5 (0.04)	3.8 (0.15)	1.1 (0.04)
	12.8	4.2 (0.07)	1.2 (0.04)	3.8 (0.12)	1.1 (0.03)	3.7 (0.13)	0.8 (0.04)	5.1 (0.04)	1.3 (0.01)
	24.2	5.9 (0.07)	1.6 (0.01)	4.3 (0.06)	1.2 (0.01)	10.0 (0.18)	1.8 (0.01)	10.1 (0.06)	1.8 (0.01)

ECi, electrical conductivity.

[a] These ratios were determined based on the Na, K, Ca and Mg concentrations presented on Tables 2 and 3, considering only the data corresponding to the harvest 2, 15 days.

[b] Ratio of means (±SE).

nutritional value; however, purslane plants exposed to moderate salinity conditions, 6.8 dS m^{-1}, showed a chemical composition similar to the control plants, except for the oil content, which was higher. This oil content increase is a positive outcome of salinity exposure, and it will be interesting to study, in the future, the detailed fatty acid profile of lipid fraction. This study also showed that purslane has a high mineral content; particularly high amounts of magnesium and potassium were found in control plants. When exposed to elevated salinity levels, potassium and calcium levels decreased and sodium and magnesium levels increased. This led to a significant increase in the Na/K, Mg/K, Na/Ca and Mg/Ca ratios and was reflected in the reduction of the growth and yield of purslane plants exposed to the more extreme salinity treatment. According to this study, purslane appears particularly well suited for cropping in areas where irrigation is necessarily carried out with water having an elevated chloride-based salinity.

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PAPER II

Molecular Cloning and expression analysis of three omega-6 desaturase genes from purslane (*Portulaca oleracea* L.)

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Molecular Cloning and expression analysis of three omega-6 desaturase genes from purslane (*Portulaca oleracea* L.)

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Abstract

Two full-length cDNA clones of *PoleFAD2* and one full-length cDNA clone of *PoleFAD6*, encoding ω-6 fatty acid desaturases, the key enzymes for the conversion of oleic into linoleic acid, were isolated from purslane (*Portulaca oleracea* L.) leaves and seeds. The deduced amino acid sequence of both isoforms of *PoleFAD2* showed higher similarities to other microsomal ω-6 desaturases then to *PoleFAD6* or other plastidial orthologues, and vice versa. Expression analysis by RT-PCR showed that all genes are expressed in all tissues of purslane tested, but higher levels of mRNA accumulation were detected in reproductive organs and cells that proliferate rapidly or store lipids. Wounding affected the levels of mRNA accumulation of both, *FAD2* and *FAD6* genes in purslane leaves, while chilling stress affected only *FAD2* transcript level. The expression patterns observed reflect the discrete roles of these genes in membrane synthesis for cell division, thylakoid development, and lipid storage or in the biosynthetic pathway for the production of signaling molecules that influence plant development or defense.

Keywords: *FAD2*, *FAD6*, Fatty acid desaturases; purslane; spatial expression; wounding, chilling stress

1. Introduction

Purslane has a long history of use for human food, animal feed and medicinal proposes. These days however, most people in modern societies limit their diet to a few cultivated vegetables so that wild plants such as purslane tend to be under-utilized. A nutritive characterization of purslane accessions conducted by Ezekwe *et al.* (1999) showed that, in spite of its genetic diversity, purslane remains one of the most abundant terrestrial vegetable sources of polyunsaturated fatty acids (PUFAs), particularly omega-3 fatty acids, and other essential nutrients potentially beneficial for humans as well as animals.

Fatty acids have many and diverse roles in plants. They are the major structural component of membrane lipids, provide a substantial reserve of free energy and serve as key precursors for the biosynthesis of messengers in signal transduction mechanisms that influence plant growth, development and responses to environmental cues (Browse and Somerville, 1991; Munnik *et al.* 1998; Somerville *et al.* 2000; Weber, 2002; Nandi *et al.* 2003). A major fraction of the fatty acids in plants are the polyunsaturated fatty acids linoleic ($C18{:}2^{\Delta 9,12}$) and α-linolenic ($C18{:}3^{\Delta 9,12,15}$). Besides their important physiological role, these fatty acids are also essential for human health and nutrition since they cannot be synthesized in the body and hence must be provide by the diet. Consequently, the desaturation of fatty acids is an important aspect in oil biochemistry since it determines the level of unsaturation and therefore the economic value of the oil (Knutzon *et al.* 1992; Mikkilineni and Rocheford, 2003). During lipid biosynthesis, the formation of the first double bound in stearic acid (C18:0) to produce the monounsaturated oleic acid ($C18{:}1^{\Delta 9}$) is catalyzed by the soluble plastidial stearoyl-ACP Δ9 desaturase. Subsequently, oleic acid is incorporated into the glycerolipids either in plastids or in the endoplasmatic reticulum (ER) membranes before being further desaturated by the membrane-bound ω-6 (Δ12) desaturases. In Arabidopsis two genes have been isolated, namely *FAD2* and *FAD6*, encoding the ER (microsomal) and the plastidial ω-6 desaturases, respectively (Falcone *et al.* 1994; Okuley *et al.* 1994). Since then, cDNAs encoding for microsomal ω-6 desaturases have been isolated from several plant species, such as soybean (Heppard *et al.* 1996), parsley (Kirsch and Hahlbrock, 1997), rape (Scheffler *et al.*1997), peanut (Jung *et al.* 2000), sesame (Jin *et al.* 2001), sunflower (Martinez-Rivez, 2001), cotton (Pirtle, 2001), tung (Dyer, 2002), and spinach (Saeki *et al.* 2004). cDNAs encoding for plastidial ω-6 desaturases have also been isolated from different species including rape (Hitz *et al.* 1994), soybean (Hitz *et al.* 1994), and spinach

(Murphy and Piffanelli, 1998). Here we report on the cloning and characterization of three purslane (*Portulaca oleracea* L.) cDNAs encoding two microsomal and one plastidial ω-6 desaturase.

Fatty acid desaturases in all organisms are subjected to several types of regulation, depending on their localization or function, and there may be requirement for different or specific desaturase activities during certain development processes in various tissues (Murphy and Piffanelli, 1998). Therefore, in order to elucidate expression patterns of these desaturases in purslane, transcript accumulation levels of these genes were investigated in different tissues of purslane plant.

Plants often encounter the abiotic stresses of low or elevated temperature, exposure to salt, drought, and, less commonly, heavy metals, as well as biotic pathogen and insect attack, sometimes simultaneously. Even with the best available land and agricultural practices, the impacts of these stresses can significantly reduce the productivity of food and fiber crops. Thus, the current situation of diminishing farm land worldwide and the potential heightened effects of global climate change on environmental, pathogen and insect stresses provide increased impetus to understand stress resistance in crop plants. More complete knowledge of fatty acid unsaturation, mobilization, and regulation processes may significantly aid the development of effective strategies for managing abiotic and biotic stresses for these plants (Upchurch, 2008). In order to elucidate the effect of chilling and wounding stresses on the expression patterns of *FAD2* and *FAD6* genes in purslane leaves, transcript accumulation levels of these genes were investigated in intact and wounded leaves of purslane plants exposed to standard (25 ºC) and low (5 ºC) growth temperatures.

2. Material and methods

2.1. Plant materials

Young expanding leaves, mature leaves, young stems, mature stems, shoot tips, roots and seeds were harvested from 1-month-old seedlings of purslane (*Portulaca oleracea* L.), grown in a growth chamber at 25 ºC under 16-h photoperiod. All samples were immediately frozen in liquid nitrogen and stored at -80 ºC for later RNA extraction. To investigate the effect of chilling and wounding stresses in the transcript accumulation level of both omega-6 FAD genes, three 1-month-old plants were transferred to a cold chamber, regulated to 5 ºC (chilling temperature) for 24 hours and other three plants were kept under regular growth temperatures 25 ºC. Two 1 cm cuts parallel to the midrib of full developed leaves were made with a sterile razor blade in plants exposed to chilling and regular growth temperatures. Samples of intact and wounded leaves were collected after 24 hours of treatment, immediately frozen in liquid nitrogen and stored at -80 ºC for later RNA extraction.

2.2. RNA extraction and RT reactions

Total RNA was isolated from *P. oleracea* tissues using a Plant RNA Purification Reagent (Invitrogen, Carlsbad CA, USA). RNA concentration was determined spectrophotometrically and verified by ethidium bromide staining in agarose gel. Total RNA was then treated with Rnase-free Dnase I (Invitrogen) and about 2 μg were used as template in first strand cDNA synthesis using Superscript^{TM} II Rnase H-Reverse Transcriptase (Invitrogen), according to manufacturer´s protocol. Unless otherwise stated, the first strand cDNA was primed off by the poly-A tail with the reverse transcription primer Oligo(18)dt primer.

2.3. PCR amplification and cloning of purslane ω-6desaturase cDNAs fragments

To amplify central cDNA fragments corresponding to purslane microsomal (designated as *PoleFAD2*) and plastidial (designated as *PoleFAD6*) ω-6 desaturases, degenerated oligonucleotide primers were designed based on reverse translation of conserved peptide sequence of *FAD2* or *FAD6* from Arabidopsis and other microsomal or plastidial orthologues, respectively, and used in RT-PCR reactions. The first-strand cDNA used was synthesized from young expanding purslane leaves and it was primed with a general AP primer (5'-GGCCACGCGTCGACTAGTACTTTTTT TTTTTTTTTTT-3'). For *PoleFAD2* gene, two oligos with sense and antisense directions were used i.e. *FAD2*fw (5´-TGGGTA/C/T/GATA/C/T/GGCA/C/T/GCAA/CGAA/GTGC/TGG-3`) and *FAD2*rv (5´-TCCATA/C/TGCA/GTG/TA/GTAA/GTGA/C/T/GGGCAT-3`), respectively. PCR was conducted with an initial Desaturation step at 94 ºC for 3 min, followed by 30 cycles of 94 ºC for 30 s, 150 s at various temperatures between 55 and 63 ºC, and 72 ºC for 1 min. For isolating cDNA fragment of *PoleFAD6* gene we used the forward primer *FAD6*fw (5`-TAC/TCCA/TTATGAA/GCCA/C/T/GTGGCG -3`) together with the standard reverse primer AUAP (5´- GGCCACGCGTCGACTAGTAC-3`). PCR was conducted in the same condition used in *PoleFAD2* amplification. cDNA amount corresponding to 200 ng total RNA was used as template, together with 200 μM of each dNTP, 20 pmol of each oligo, 2 U of DNA polymerase (Expand^{TM} High Fidelity, Boehring, Mannheim) in a 50 μl reaction volume. All amplifications were achieved in a Corbett Research cooled/gradient Palm Cycler. PCR products were isolated from agarose and purified using QIAquick^R PCR Purification Kit (Qiagen, Germany), before cloned into The PTZ57R/T vector (Fermentas, Lithuania) according to the manufacture's instructions. The resultant clones were sent to Eurofins MWG GmbH (Germany) for sequencing on both strands from plasmids using M13(21)fw/M13(29)rv primers.

2.4. Rapid amplification of cDNA ends (RACE)

For amplification of the unknown 3'and 5'-ends of the above cDNA fragments, the 3'and 5´RACE methods were used according to protocols described elsewhere [26]. For 3'RACE, the first-strand cDNA was primed with a general AP primer. Based on sequence information of the central cDNA fragments, specific forward primers were designed. For *PoleFAD2* we used the primer *FAD2* 3'RACEfw (5'-ATGCCACACTACCACGCAATGGA-3') together with the general AUAP primer (5'-GGCCACGCGTCGACTAGTAC-3'). For 5'RACE, he synthesis of first-strand cDNAs of *PoleFAD2* and *PoleFAD6* were conducted using the reverse specific primers *FAD2*rv1 (5'-CCGTAGATGGGCCAGGCTAAGTAGTT-3') and *FAD6* rv1 (5'-CCACTTGTCTGATTGGTTG AATG-3'), respectively. First-strand cDNAs were dC-tailed and amplified using the nested *PoleFAD2*-specific reverse primer *FAD2*rv2 (5'-GGATGGCTGCGATGGTGAGATCGTAAA-3') followed by *FAD2*rv3 (5'-GGATGGCTGCGATGGTGAGATCGTAA-3') and *FAD2*rv4 (5'-GGATGTAGGTTGTGGCGAGATAA-3'); and the *PoleFAD6*-specific primers *FAD6* rv2 (5'-GC CGTATGGTGGACTATTGTGAAC-3'), followed by *FAD6*rv3 (5'-GGTAACCTAACCTCGGC ATGAGCCAGAAT-3', *FAD6*rv4 (5'-CCACAATAACCAGTGAGCTATTGA-3') and *FAD6*rv5 (5'-GGACCATAGCCAAAAATCATAGCTTTA-3'), in combination with AUAP. To amplify the full length *PoleFAD2* and Pole *FAD6* cDNAs, the sense/antisense primer pairs *FAD2*flfw (5'-ATAGGATCCAGGATGGGTGCAGGTGG-3)/ *FAD2*flrv (5'-AGCTTGTTGTTGTACCAGTGG ATACCTTT-3') and *FAD2*flfw (5'-ATAGGATCCAGGATGGGTGCAGGTGG-3)/ *FAD2*flrv (5'-AGCTTATTATTATACCAGAGGATGCCTTT-3') for *PoleFAD2*-s and *PoleFAD2*-l, respectivelly, and *FAD6*flfw (5'-ATAGGATCCGGAATGGCTTCCACCATTTCAGACT-3')/ *FAD6*flrv (5'-TGCGAATTCCTAAGCATAATCAGGCATTACTCTT-3') were used, respectively, designed from the respective 5'- and 3'-UTRs. These fragments were cloned and sequenced, as above, to confirm identity to previous cloned cDNA fragments.

2.5. Transcript analysis by Semi-quantitative Real Time PCR

The steady state levels of *PoleFAD2* and *PoleFAD6* transcripts were estimated in various purslane tissues. Two sets of primers were design to amplify fragments of proximally 200 bp of conserved regions of each target gene.*FAD2-1* RT-PCR fw (5'-GGGTTCGTCCAGGGCTGTGTGCT-3')/*FAD2-1* RT-PCRrv (5'-CCACTGGAGGCCGGACTTTCGTTT-3') and *FAD2-2* RT-PCR fw (5'-GGCTACATCCAAGGTTGCATCTT-3')/*FAD2-2* RT-PCRrv (5'-CCACTTCATGCCATCCT TCTTCTT-3') primer sets were used for real-time semi-quantitative PCR analysis of both isoforms of the *FAD2* gene, designated as mentioned above, and for the *PoleFAD6* gene the *FAD6* RT-PCR fw (GCCGTGCCAGAAGGGTTTCGGTA-3') and *FAD6* RT-PCRrv (CACCTTCTTAGGAAGG GAACTGAT-3') primers, both designed from the coding region. As a control, a part of the coding region of GAPDH gene was amplified with the specific forward primer GAPDHfw (5'-CACGGCCACTGGAAGCA-3'), and the reverse GAPDHrv (5'-TCCTCAGGGTTCCTGATGCC -3'). PCRs were preformed using equal amounts of templates (25 ng cDNA) and gene specific or GAPDH primers and carried out for different number of cycles in order to optimize reproducibility and ensure that reactions remained in log-linear range.

The expression patterns of the two omega-6 FAD genes, *FAD2-2* and *FAD6*, were examined by semi-quantitative real time PCR assay in intact and wounded leaves of plants exposed to normal (control, 25ºC) and low temperatures (5 ºC). The same set of primers, mentioned above, were used in these assays. PCRs were preformed using equal amounts of template. Relative expression of target genes in purslane tissues was calculated using the efficiency calibrated Model (Pfaffl, 2001). The RT-qPCR of reference and target gene transcripts in cDNA samples were conducted in an ABI model 7500 thermocycler (Applied Biosystems). All samples were amplified in duplicates in three independent assays under the following conditions: 95°C for 10 min for 1 cycle, followed by 40 cycles of 95°C for 15 s and 1 min at 60 °C. The PCR products for each primer set were also subjected to melt-curve analysis. RT-qPCR results were analysed with the sequence detection software SDS version 1.1 (Applied Biosystems). A standard fluorescence threshold was set to a ΔRn of 0.5 on the log fluorescence scale to determine the fractional cycle number (Ct value). For each cDNA sample, relative expression levels of each protein coding gene were normalized by reference to the GAPDH gene assay. The transcript abundance ratio of the target gene to reference gene was determined by equation 1:

$$\text{Relative expression} = (E_{\text{ref}})^{\Delta\text{Ct ref}} / (E_{\text{target}})^{\Delta\text{Ct target}} \qquad \text{Equation 1(Pfaffl, 2001)}$$

where E_{ref} and E_{target} are the efficiencies of the primers for the reference and the target gene, respectively, and ΔCt_{ref} and ΔCt_{target} are the difference between the mean Ct value of reference and target genes for the control plants and the plants exposed to temperature or/and wounding treatments, respectively. The RT-qPCR protocol was optimized by determining the optimal primer concentration and primer efficiency. The RT-qPCR products were separated by 1% agarose gel electrophoresis. The RT-qPCR fragments were cloned in *E. coli* and sequenced by Eurofins MWG GmbH (Germany), confirming the identity of the amplified products.

2.6. Sequence analysis

Nucleotide sequences from cDNA clones and deduced amino acid sequences were identified by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Predictions of open reading frames (ORFs) and theoretical molecular weights of deduced polypeptides were made using the Protein property calculator (http://www.basic.northwestern.edu/biotools/proteincalc.html). Sequence comparation was conducted using the Clustalw program.

Hydropathy analysis was made by using the Protein Sequence Analysis Software (I.W. Palmer, Gaithersburg, MD, USA) and transmembrane regions were predicted by the TMHMM server ver.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Predictions of subcellular localization of the deduced polypeptides were conducted by using PSORT (http://www.psort.nibb.ac.jp./form.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) algorithms.

Amino acid multiple alignments were made with the ClustalW program under default parameters. A phylogenetic tree was constructed using the neighbor-joining algorithm included in the ClustalW program. The tree was visualized using the TreeView program.

3. Results and discussion

In the plant fatty acid desaturation pathway, the first step is catalyzed by stearoyl-ACP desaturase to convert stearic acid into oleic acid. In Arabidopsis, microsomal and plastidial ω-6 fatty acid desturases, *FAD2* and *FAD6*, are the two enzymes responsible for converting oleic acid to linoleic acid through the eukaryotic and the prokaryotic pathway, respectively. Further desaturation of linoleic acid to produce trieonic fatty acids is catalyzed by ω-3 fatty acid desaturases. Therefore, despite the key role of ω-6 desaturases for the production of polyunsaturated fatty acids, which have several important roles in both plant development and defense responses (McConn and Browse, 1996, Mconn *et al.* 1997), they alternatively possess crucial role to oleic acid content of vegetable oil, which is important factor for oil quality.

3.1. Cloning and sequence analysis of purslane ω-6 desaturases

In the present study, using a PCR-based approach, we have cloned three distinct ω-6 fatty acid desaturase cDNAs from purslane. Two full length *PoleFAD2* clones were obtained; one was isolated from the leaves and the other from seeds, hereby designated by *PoleFAD2-2* and *PoleFAD2-1*. *PoleFAD2-2* revealed a putative 1151-nucleotide ORF encoding a predicted polypeptide of 383 amino acid residues of calculated molecular mass 44.0 kDa. *PoleFAD2-1* revealed a putative 1148-nucleotide ORF encoding a predicted polypeptide of 381 amino acid residues of calculated molecular mass 43.8 kDa. One full-length *PoleFAD6* cDNA clone was isolated from young expanding purslane leaves, hereby designated as *PoleFAD6*-l. *PoleFAD6*-l revealed a putative 1374-nucleotide ORF encoding a predicted polypeptide of 457 amino acid residues of molecular mass 52.9 kDa.

Hydropathy analysis of the three genes showed that the encoded polypeptides contained four putative membrane-spanning domains at amino acid residues 54-76, 81-103, 177-199, and 246-268, for *PoleFAD2-2*, residues 54-76, 81-103, 177-199 and 249-271, for *PoleFAD2-1*, and residues 126-155, 159-181, 285-304 and 308-327 for *PoleFAD6*. The prediction of four putative transmembrane regions supports the notion that all three desaturases are membrane bound.

Inspection of the sequences revealed the presence of eight highly conserved histidine residues in three separated clusters, i.e. residues 104-109 (HXXXH), 140-144 (HXXHH) and 314-318 (HXXHH) for both isoforms of *PoleFAD2* and residues 181-185, 217-221 and 377-381 for *PoleFAD6* (Fig. 1B) with the consensus sequence: $HX_{(3-4)}HX_{(20-50)}HX_{(2-3)}HHX_{(100-200)}HX_{(2-3)}HH$. (Fig. 1). These conserved motifs are found in all membrane bound desaturases and contribute to an iron-binding site required for enzyme activity (Okuley *et al,*. 1994; Los and Murata, 1998; Murphy and Piffanelli, 1998; Tao *et al.* 2006).

Based on conserved amino-acid sequences of microsomal (*FAD2*) and plastidial (*FAD6*) ω-6 desaturase from different plant species, degenerated oligonucleotide primers were designated corresponding to each desaturase. PCRs employing these primer pairs with purslane cDNA from leaves and seeds as template generated fragments with the approximate expected sizes. Sequence and BLAST searches of both the nucleotide and deduced amino acid sequences suggested them to be central parts of the respective genes. To clone the full-length cDNAs, 5' and 3' RACE reactions were conducted. The 5'-RACE PCRs generated a fragment of around 400 bp for both *PoleFAD2* isoforms or 445 bp for the *PoleFAD6* gene, and the 3`RACE reaction generated fragments of 348, 350 bp or 600 bp, respectively. BLAST searches of the deduced amino acid sequences revealed correspondence to the expected genes. Sequence comparison of the 5'- and 3-ends with the central parts of the genes indicated that the overlapping regions match perfectly, suggesting that these sequences represent the missing parts of the gene. Based on the above sequence data, primers were designed from the 5'- and 3'-UTRs of both genes and the full-length cDNAs were amplified, cloned and sequenced, revealing 100 % identity to the expected sequences. Two full length *PoleFAD2* clones were obtained; one was isolated from the leaves and the other from seeds, hereby designated by *PoleFAD2-2* and *PoleFAD2-1*. *PoleFAD2-2* revealed a putative 1151-nucleotide ORF encoding a predicted polypeptide of 383 amino acid residues of calculated molecular mass 44.0 kDa. *PoleFAD2-1* revealed a putative 1148-nucleotide ORF encoding a predicted polypeptide of 381 amino acid residues of calculated molecular mass 43.8 kDa. Hydropathy analysis of both genes showed that the encoded polypeptides contained four putative membrane-spanning domains at amino acid residues 54-76, 81-103, 177-199, and 246-268, for *PoleFAD2-2* and 54-76, 81-103, 177-199 and 249-271, for *PoleFAD2-1*, respectively. Inspection of the sequences revealed the presence of eight highly conserved histidine residues in three separated clusters, i.e.104-109, 140-144 and 314-318 for both isoforms (Fig. 1A). These residues are shown to be necessary for the function of all membrane-bound fatty acid desaturases (Tao *et al.* 2006). These invariant residues were arranged in three histidine boxes (HXXXH, HXXHH, and HXXHH) with conserved spaces between them (Fig.1). These histidine boxes participate in the formation of active sites with iron and are conserved in all omega-6 type fatty acid desaturases (Los and Murata, 1998).

Figure 1

One full-length *PoleFAD6* cDNA clone was isolated from young expanding purslane leaves, hereby designated as *PoleFAD6*-l. *PoleFAD6*-l revealed a putative 1374-nucleotide ORF encoding a predicted polypeptide of 457 amino acid residues of molecular mass 52.9 kDa. Hydropathy analysis revealed four trans-membrane regions of the predicted at amino acid residues 1*26*-155, 159-181, 285-304 and 308-327. The highly conserved histidine boxes were also present at three separate clusters of residues 181-185, 217-221 and 377-381 (Fig. 1B).

Blast searches showed high similarities of the predicted *PoleFAD2* and *PoleFAD6* amino acid sequences to several orthologues desaturases available in the GenBank database. *PoleFAD2-2* and *PoleFAD2-1* revealed 63-72% identity (68-83 % similarity) and 67-82 % (73-82 % similarity) to other *FAD2*, respectively; *PoleFAD6* showed 64-70% identity (82-85% similarity) to *FAD6* proteins from different plant species. The sequence identity or similarity between *PoleFAD2*-l and *PoleFAD2*-s was higher, 87% identity; and between these two isoforms of *FAD2* gene and *PoleFAD6* was much lower (17 or 19 %, respectively), indicating the high phylogenetic divergence of these three ω-6 desaturases.

To predict whether signal or plastidial transit peptides are present in the N-terminal regions of the above polypeptides, two different algorithms (PSORT and TargetP) for amino acid sequence analysis were introduced. For the *PoleFAD2* polypeptide no indication of plastidial leader peptide was detected at the N-terminus, whereas a *FAD2* ER aromatic amino acid-enriched retrieval signal (-YNNKSL/ -YNNKL) was present at the C-terminus (Fig. 1A; Table 1), which is both necessary and sufficient for maintaining localization in the ER (McCartney *et al.* 2004). Analysis of *PoleFAD6* polypeptide made it possible to predict a putative chloroplast transit peptide sequence of 65 amino acids at the N-terminus (Fig. 1B; Table 1). This N-terminal sequence also had several characteristics of plastidial transit peptides, including a high content of hydroxylated residues (Ser, Thr and Tyr), a low content of acidic residues and the conserved N-terminal Met-Ala dipeptide (Liu *et al.* 1999a,b). Based on multiple alignments of their deduced amino acid sequences and phylogenetic analysis it was shown that both isoforms of *PoleFAD2* were highly similar to other ER localized ω-6 fatty acid desaturases, whereas *PoleFAD6* was highly homologous to other plant ω-6 fatty acid desaturases that have putative plastidial transit peptides.

Table1

To elucidate phylogenetic relationships of *PoleFAD2* and *PoleFAD6* genes, their deduced amino acid sequences were aligned with other homologous plant ω-6 desaturase sequences and a N-J tree was constructed. As shown in Figure 2, the analysis revealed that the *FAD6* gene family, including *PoleFAD6*, forms a tight cluster of more related sequences to each other than to the members of *FAD2* subgroups. These observations support the hypothesis that the evolutionary process of *FAD2* might be different from that of *FAD6* genes (Seffens *et al.* 1990; Jin *et al.* 2001). In addition, the relative high level of divergence within the *FAD2* group observed implies that certain genes may have evolved differently, although to further confirm details in phylogenetic relationships within this gene family more data are required. Nevertheless, the high phylogenetic divergence of *FAD2* compared to that of *FAD6* is further supported by the identification of diverged *FAD2* enzymes, in non-traditional crop plants species, synthesizing a variety of structurally unrelated fatty acids (Shanklin and Cahoon, 1998).

Figure 2

3.2. Expression of ω-6 desaturase genes in different tissues

To determine the steady-state level *PoleFAD2* or *PoleFAD6* gene expression in different tissues of purslane, RT-PCR analysis was carried out with total RNA extracted from young expanding leaves, mature leaves, young stems (tips), stems, root, root tips and seeds (Fig.3). Similar to *Arabidopsis FAD2*, maize *FAD2,* soybean *FAD2-2*, cotton *FAD2-2*, *FAD2-3*, *FAD2-4*, and sunflower *FAD2-2* and *FAD2-3*, *PoleFAD2-2* is expressed in vegetative tissues and developing seeds (Fig. 3). On the other hand, like cotton, soybean and sunflower *FAD2-1*, *PoleFAD2-1* is only expressed in developing seeds (Fig. 3). The *PoleFAD6* gene was expressed in all plant tissues (Fig. 3), including non-photosynthetic tissues, such as seed and root, with highest expression in young developing leaves and stems. The *FAD6* gene has been shown to be expressed only in photosynthetic tissues in *Arabidopsis* (Falcone *et al.* 1994; Miquel and Browse, 1992).

PoleFAD2-1 was exclusively involved in storage lipids desaturation in seed, supported by its exclusive expression in seeds. *PoleFAD2-2* may be partially responsible for desaturation of storage lipids in developing seeds and also be involved in membrane lipids desaturation in root, leaf and stem. *PoleFAD6* gene may have a more extensive role in membrane lipid desaturation in photosynthetic tissues such as leafs and stems, but also, to a lesser extent in the seed storage lipid desaturation.

Figure 3

3.3. Expression of ω-6 desaturase genes in plants under stress

Abiotic stress-induced changes in the fatty acid composition of plant membrane lipids mainly occur through the regulated activities of fatty acid desaturases. A large body of research suggests that temperature regulates fatty acid desaturase expression at both the transcriptional and post-transcriptional levels (Upchurch 2008). In order to study the *P. oleracea* response to low temperature and wounding treatments, the mRNA transcript accumulation pattern of the two omega-6 genes *PoleFAD2-2* and *PoleFAD6* were followed in control and stressed plants by semi-quantitative real-time PCR analysis. An increase of mRNA transcript level of both genes was detected in plants submitted to the treatments compared with the control (Figs. 4 and 5). *PoleFAD2-2* increase in relative expression was detected in chilled and wounded leaves of purslane (Figs. 4 and 5). No cumulative effect of chilling and temperature stresses was observed in the expression levels of this gene, but an increase in expression was also detected when both treatments were applied (Figs. 4 and 5). *PoleFAD6* relative expression was not significantly affected by chilling temperatures, but an increase of expression was detected for the wounding treatment (Figs. 4 and 5).

Figure 4, Figure 5

In many plant species, the content of di- and tri-unsaturated fatty acids increases under cold stress (Li *et al.* 2006, Okuley *et al.* 1994). However, the increase in di-unsaturated fatty acids has been shown to be due to post-transcriptional/post translational modifications on the enzyme rather than to increases in its mRNA levels (Heppard *et al.* 1996;. Falcone *et al.* 2004, Tang *et al.* 2005). However, in our study, we observed an increase in the expression of *PoleFAD2-2* gene in response to chilling temperature and wounding stresses (Figs. 4 and 5), which may indicate that an increase in mRNA levels occurred in response to the applied treatment. It is possible that temperature regulates fatty acid desaturase expression at both the transcriptional and post-transcriptional levels in this plant. Similar results were reported by Kargiotidou *et al.* (2008) where an increase in mRNA levels of a membrane modifying enzyme were also detected in cotton (*Gossypiun hirsutum*) plants under cold and wound-induced stresses. It was also reported that *FAD2* expression under chilling temperatures was also light–dependent. It is possible that temperature regulates fatty acid desaturase expression at both the transcriptional and post-transcriptional levels in these plants.

In the plant fatty acid desaturation pathway, the first step is catalyzed by stearoyl-ACP desaturase to convert stearic acid into oleic acid. Plant microsomal and plastidial ω-6 fatty acid desturases, *FAD2* and *FAD6* in Arabidopsis, are the two enzymes responsible for converting oleic acid to linoleic acid through the eukaryotic and the prokaryotic pathway, respectively. Further desaturation of linoleic acid to produce trieonic fatty acids is catalyzed by ω-3 fatty acid desaturases. Therefore, despite the key role of ω-6 desaturases for the production of polyunsaturated fatty acids, which have several important roles in both plant development and defense responses (McConn and Browse, 1996, Mconn *et al.* 1997), they alternatively possess crucial role to oleic acid content of vegetable oil, which is important factor for oil quality.

In the present study, using a PCR-based approach, we have cloned three distinct ω-6 fatty acid desaturase cDNAs from purslane, designated as *PoleFAD2*-l, *PoleFAD2*-s and *PoleFAD6*. *PoleFAD6* gene was highly divergent in the sequence structure, compared to the two isoforms of *FAD2* gene, and either of them resembles more closely ω-6 desaturase genes from different species than each other. Based on multiple alignments of their deduced amino acid sequences and phylogenetic analysis it was shown that both isoforms of *PoleFAD2* were highly similar to other ER localized ω-6 fatty acid desaturases, whereas *PoleFAD6* was highly homologous to other plant ω-6 fatty acid desaturases that have putative plastidial transit peptides.

Besides the high homology to other published sequences found, eight histidines in three separate clusters were also detected in both genes with the following consensus sequence: $HX_{(3-4)}HX_{(20-50)}HX_{(2-3)}HHX_{(100-200)}HX_{(2-3)}HH$. These conservative motifs found in all membrane bound desaturase and contributes to an iron-binding site (Okuley *et al,*. 1994; Los and Murata, 1998; Murphy and Piffanelli, 1998). The prediction of four putative transmembrane regions for *PoleFAD2*-l, *PoleFAD2*-s and *PoleFAD6* polypeptides also support the notion that all three desaturases are membrane bound. Further sequence analysis showed that both isoforms of *FAD2* amino acid sequence have an aromatic amino acid-enriched sequence at the C-terminus (-YGNKS/ -YNNTL) that has been found to act as an ER retrieval signal (McCartney *et al.* 2004). As opposed, *PoleFAD6* possessed a putative N-terminus transit peptide of 65 amino acids capable of moving the molecule into the plastids (Fig. 1).

Phylogenetic analysis revealed that the *FAD6* gene family, including *PoleFAD6*, forms a tight cluster of more related sequences to each other than to the members of *FAD2* subgroups. These observations support the hypothesis that the evolutionary process of *FAD2* might be different from that of *FAD6* genes (Seffens *et al.* 1990; Jin *et al.* 2001). In addition, the relative high level of divergence within the *FAD2* group observed implies that certain genes may have evolved differently, although to further confirm details in phylogenetic relationships within this gene family more data are required. Nevertheless, the high phylogenetic divergence of *FAD2* compared to that of *FAD6* is further supported by the identification of diverged *FAD2* enzymes, in non-traditional crop plants species, synthesizing a variety of structurally unregulated fatty acids (Shanklin and Cahoon, 1998).

Both ER and plastidial ω-6 desaturases were expressed in all tissues of purslane studied (Fig.3), reflecting the crucial roles of these enzymes in fatty acid desaturation. The expression of the plastidial *PoleFAD6* was not restricted to photosynthetic tissues, as it was shown by its expression in root and seed tissues. *PoleFAD2-2* and *PoleFAD6* genes shared, qualitatively, almost similar pattern of expression, with the highest transcript levels observed in actively developing young organs, i.e. expanding leaves and shoot tips (young stems), most likely to furnish the demand of polyunsaturated fatty acids in membrane biosynthesis. *PoleFAD2-2* and to a lesser extent *PoleFAD6* were also up-regulated in seeds (Fig. 3). Tao *et al.* (2006) also reported a high level of expression in immature embryos (seeds) and lower level of expression of *FAD2* gene in photosynthetic tissues (leaves, stems and root) in maize (*Zea maize*).

Delta-12 fatty acid desaturase of *Arabidopsis* is encoded by a single *FAD2* gene in all kinds of tissues while there are two different cDNA sequences in soybean, *FAD2-1* and *FAD2-2*, encoding microsomal delta-12 fatty acid desaturase. The *FAD2-1* gene is strongly expressed in developing seeds only, while *FAD2-2* gene is constitutively expressed in both vegetative tissues and developing seeds (Heppard *et al.* 1996). In *Olea europaea*, the desaturase encoded by *FAD2-1* is possibly responsible for the desaturation of reserved lipids in young seeds, while the desaturase encoded by *FAD2-2* may be involved mainly in storage lipids desaturation in mesocarp and maturing seeds (Hernandez *et al.* 2005). In cotton and sunflower, this enzyme is coded by multiple genes expressed in different tissues (Liu *et al.* 1999a,b; Martinez-Rivaz *et al.* 2001; Pirtle *et al.* 2001). Similar to *Arabidopsis FAD2*, maize *FAD2* soybean *FAD2-2*, cotton *FAD2-2*, *FAD2-3*, *FAD2-4*, and sunflower *FAD2-2* and *FAD2-3*, *Portulaca FAD2-2* is expressed in vegetative tissues and developing seeds

(Fig.3). In the other hand, like cotton, soybean and sunflower *FAD2-1*, *Portulaca FAD2-1* is only expressed in developing seeds (Fig.3). Falcone *et al.* (1994) observed a progressive decrease of *FAD6* expression during later stages of *Arabidopsis* plant growth in photosynthetic tissues, which may indicate a reduced demand for membrane biogenesis in fully expanded leaves, compared with young expanding leaves. Miquel and Browse (1992) also refer that gene alterations involving *FAD6* gene were exclusively expressed in photosynthetic tissues. *Portulaca FAD6* gene was expressed in all plant tissues (Fig. 3), including non photosynthetic tissues, such as seed and root, with higher expression present in young developing leaves and stems. The expression pattern of these fatty acid desaturase genes suggests different roles on the lipid desaturation process in purslane tissues. *PoleFAD2-1* was exclusively involved in storage lipids desaturation in seed, supported by its exclusive expression on seeds. *PoleFAD2-2* may be partially responsible for desaturation of storage lipids in developing seeds and also be involved in membrane lipids desaturation in root, leaf and stem. *PoleFAD6* gene may have a more extensive role in membrane lipid desaturation in photosynthetic tissues such as leafs and stems, but also, to a lesser extent in the seed storage lipid desaturation.

Abiotic stress-induced changes in the fatty acid composition of plant membrane lipids mainly occur through the regulated activities of fatty acid desaturases. A large body of research suggests that temperature regulates fatty acid desaturase expression at both the transcriptional and post-transcriptional levels (Upchurch 2008). In order to study the *P. oleracea* response to low temperature and wounding treatments, the mRNA transcript accumulation pattern of the two omega-6 genes *PoleFAD2-2* and *PoleFAD6* were followed in control and stressed plants. In many plant species, the content in di- and tri-unsaturated fatty acids increases under cold stress. However, the increase in di-unsaturated fatty acids was not attributed to a rise in mRNA levels of the enzyme. A study on *FAD2-3* from soybean (Li *et al.* 2006) and Arabidopsis *FAD2* gene (Okuley *et al.* 1994) were in accordance with the general acceptance indicating that the increase in di-unsaturated fatty acid composition under cold stress is due to post-transcriptional/post translational modifications on the enzyme rather than to increases in its mRNA levels (Heppard *et al.* 1996;. Falcone *et al.* 2004, Tang *et al.* 2005). However, in our study, we observed an increase in the expression of *PoleFAD2-2* gene in response to chilling temperature and wounding stresses (Figs. 4 and 5) which may indicate that an increase in mRNA levels occurred in response to the applied treatment. Similar results were reported by Kargiotidou *et al.* (2008) where an increase in mRNA levels of a membrane modifying enzyme were also detected in cotton (*Gossypiun hirsutum*) plants under cold and wound-induced stresses. It was also reported that *FAD2* expression under chilling temperatures was also light –dependent. It is possible that temperature regulates fatty acid desaturase expression at both the transcriptional and post-transcriptional levels in these plants.

Wounding enhanced the accumulation of *PoleFAD6* gene mRNA, showed by the increase in expression levels of the gene in wounded leaves of purslane in relation to intact leaves of plants grown at 25 ºC (Figs. 4 and 5). An increase of expression was also detected in purslane plants exposed to both, temperature and chilling treatments (Fig. 5). Since no significant alteration in expression levels was detected when only chilling stress was applied, we considered that the increase of expression levels when both treatments were applied was due to the wounding stress and not the temperature.

In summary, two microsomal and one plastidial omega-6 fatty acid desaturase genes were isolated from *Portulaca oleracea* L. tissues. The deduced amino acid sequence of both isoforms of *PoleFAD2* showed higher similarities to other microsomal ω-6 desaturases then to *PoleFAD6* or other plastidial orthologues, and vice versa. The expression pattern of these fatty acid desaturase genes suggests different roles on the lipid desaturation process in purslane tissues. *PoleFAD2-1* was exclusively involved in storage lipids desaturation in seed, supported by its exclusive expression on seeds. *PoleFAD2-2* may be partially responsible for desaturation of storage lipids in developing seeds and also be involved in membrane lipids desaturation in root, leaf and stem. *PoleFAD6* gene may have a more extensive role in membrane lipid desaturation in photosynthetic tissues such as leafs and stems, but also, to a lesser extent in the seed storage lipid desaturation. Wounding affected the expression of both genes, *PoleFAD6* and *PoleFAD2-2*, in purslane leaves, while chilling temperatures affected only *PoleFAD2-2* relative expression. Contrary to the general acceptance that the increase in the levels di-unsaturated fatty acid composition under cold stress is due to post-transcriptional/post translational modifications on the enzyme rather than to increases in its mRNA levels, we did observe an increase in transcriptional levels of *PoleFAD2-2* gene in the leaves of chilled purslane plants. It is possible that temperature regulates fatty acid desaturase expression at both the transcriptional and post-transcriptional levels in this plant.

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Tables

Table 1. Description of three full-length cDNAs encoding omega-6 fatty acid desaturases from *Portulaca oleracea* L.

Name	Full-length cDNA (bp)	ORF (bp)	Encoded amino acid	Protein molecular mass (KDa)	Isoelectric point	Predicted subcellular location
PoleFAD2-2	1232	1151	383	44.0	9.2	ER
PoleFAD2-1	1223	1148	381	43.8	8.7	ER
PoleFAD6	1573	1374	457	52.9	9.2	Chloroplast

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Fig. 1. (A) Alignment of the deduced amino acid sequences determined for *PoleFAD2-1* (FJ472350), *PoleFAD2-2* (EU872254) and previously published microsomal ω-6 fatty acid desaturases from *Arabidopsis thaliana* (AAG51042), *Brassica napus* (AAF78778), *Vernicia fordii* (AAN87573), *Gossypium hirsutum* (AAL37484), *Sesamum indicum* (AAF80560), *Nicotiana tabacum* (AY660024), *Helianthus annuus* (AAL68981), *Arachis duranensis* (AAF82294), *Olea europae* (AAL93620), and *Spinacea oleracea* (BAC22091). (B) Alignment of the deduced amino acid sequences determined for *PoleFAD6* (EU376530) and previously published plastidial ω-6 fatty acid desaturases from *A. thaliana* (P46312), *B. napus* (P48627), *Olea europaea* (AY772187), *G. max* (P48628) and *S. oleracea* (P48629). The arrowed indicates a putative signal peptide cleavage site of chloroplast transit peptide sequence. The square indicates a putative ER aromatic amino acid-enriched retrieval signal. Numbers in parentheses refer to GenBank accession numbers. Identical or similar amino acids are shaded black or grey, respectively. The three characteristic histidine boxes encountered in *FAD2* or *FAD6* subfamilies are underlined. Numbers to the right indicate the position of amino acids.

Fig. 2. Phylogenetic relationships between deduced amino acid sequences from *PoleFAD2* and *PoleFAD6* cDNAs and other plant microsomal (*FAD2*) or Plastidial (*FAD6*) oleate desaturases. At, *Arabidopsis thaliana*; Bn, *Brasica napus*; Ds, *Descurainia Sophia*; Oe, *Olea europea*; Gm, G*lycine. max*; So, *Spinacea oleracea*; Ha, H*elianthus. annuus*; Ad, *Arachis duranensis*; Nt, *Nicotiana Tabacum*; Si, *Sesamum indicum*; Vf, *Vernicia fordii*; Gh, *Gossypium hirsutum*; Po, *Portulaca oleracea*. The GenBank accession numbers of the above sequences are shown in Fig. 1. The tree was constructed by using the Neighbor-Joining algorithm.

Fig. 3. Real-time PCR spatial analysis of *PoleFAD2-1*, *PoleFAD2-2* and *PoleFAD6* genes. First strand cDNAs were synthesized from total RNA extracted from young expanding leaves (YL), full-expanded mature leaves (ML), young stems (YSt), stems (S), roots (R), root tips (Rt) and seeds (Sd). To ensure equal amounts of template, GAPDH was used as a reference gene.

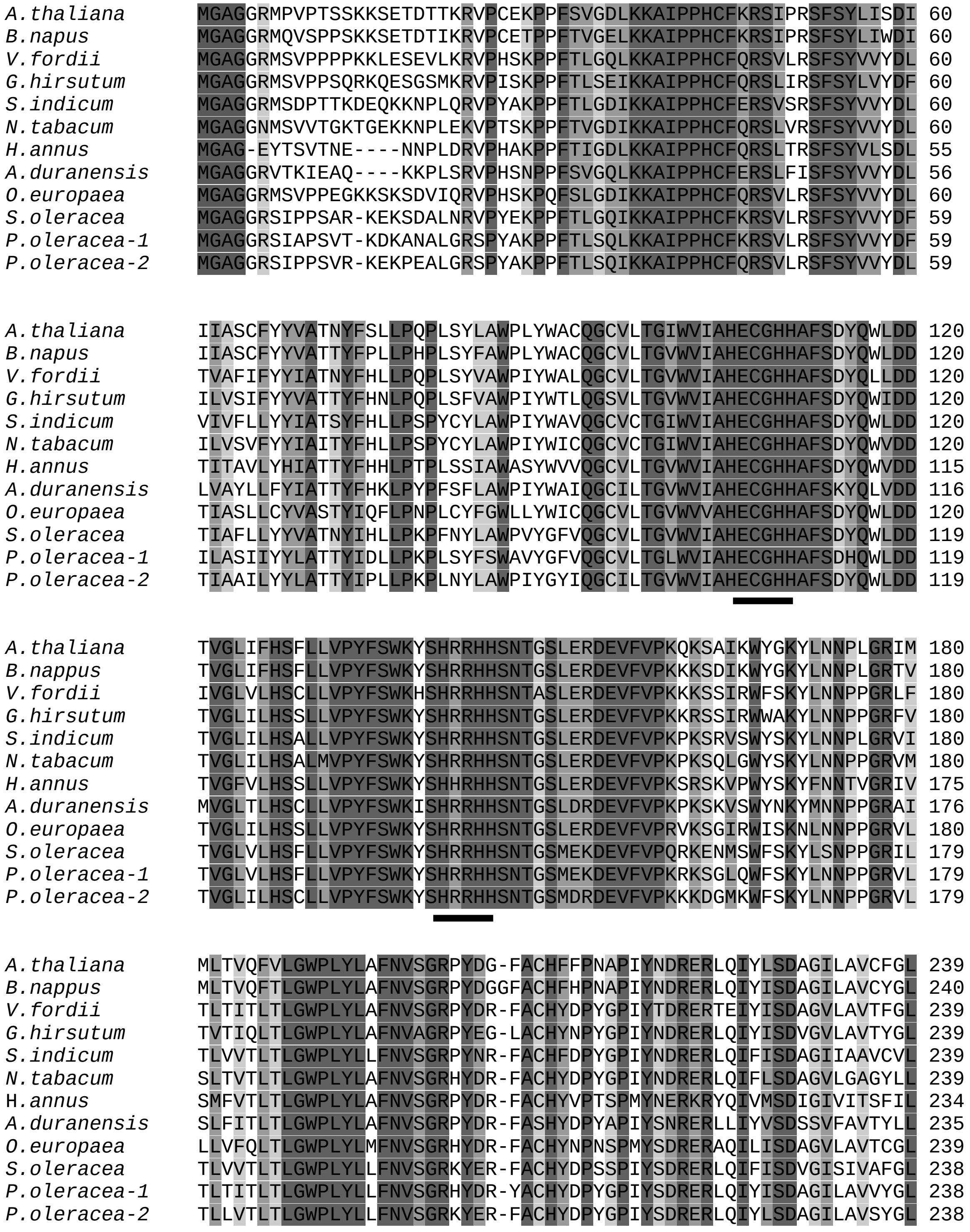
Fig. 4. Real-time PCR analysis of *PoleFAD2-2* and *PoleFAD6* genes in leaves of stressed purslane plants. First strand cDNAs were synthesized from total RNA extracted from intact (C) and wounded (W) leaves of plants grown at 25 ºC and plants exposed to chilling temperature, 5 ºC, for 24 h (T and T+W, respectively).

Fig. 5. Relative expression of target genes in leaves of *P. oleracea* plants subjected to chiling and wounding treatments. Assays of transcript levels for each target gene were normalized by GAPDH gene expression for ΔCt value calculation. Target gene expression level in plants exposed to the treatments was also normalized by the expression level of each corresponding gene in the leaves of control plants. The continuous line corresponds to a relative expression level of 1, in which the target gene expression from the plant exposed to the wounding and chilling treatments equals the target gene expression in the control plants.

Figures

Figure 1

(A)



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A.thaliana      MGAGGRMPVPTSSKKSETDTTKRVPCEKPPFSVGDLKKAIPPHCFKRSIPRSFSYLISDI 60
B.napus         MGAGGRMQVSPPSKKSETDTIKRVPCETPPFTVGELKKAIPPHCFKRSIPRSFSYLIWDI 60
V.fordii        MGAGGRMSVPPPPKKLESEVLKRVPHSKPPFTLGQLKKAIPPHCFQRSVLRSFSYVVYDL 60
G.hirsutum      MGAGGRMSVPPSQRKQESGSMKRVPISKPPFTLSEIKKAIPPHCFQRSLIRSFSYLVYDF 60
S.indicum       MGAGGRMSDPTTKDEQKKNPLQRVPYAKPPFTLGDIKKAIPPHCFERSVSRSFSYVVYDL 60
N.tabacum       MGAGGNMSVVTGKTGEKKNPLEKVPTSKPPFTVGDIKKAIPPHCFQRSLVRSFSYVVYDL 60
H.annus         MGAG-EYTSVTNE----NNPLDRVPHAKPPFTIGDLKKAIPPHCFQRSLTRSFSYVLSDL 55
A.duranensis    MGAGGRVTKIEAQ----KKPLSRVPHSNPPFSVGQLKKAIPPHCFERSLFISFSYVVYDL 56
O.europaea      MGAGGRMSVPPEGKKSKSDVIQRVPHSKPQFSLGDIKKAIPPHCFQRSVLRSFSYVVYDL 60
S.oleracea      MGAGGRSIPPSAR-KEKSDALNRVPYEKPPFTLGQIKKAIPPHCFKRSVLRSFSYVVYDF 59
P.oleracea-1    MGAGGRSIAPSVT-KDKANALGRSPYAKPPFTLSQLKKAIPPHCFKRSVLRSFSYVVYDF 59
P.oleracea-2    MGAGGRSIPPSVR-KEKPEALGRSPYAKPPFTLSQIKKAIPPHCFQRSVLRSFSYVVYDL 59

A.thaliana      IIASCFYYVATNYFSLLPQPLSYLAWPLYWACQGCVLTGIWVIAHECGHHAFSDYQWLDD 120
B.napus         IIASCFYYVATTYFPLLPHPLSYFAWPLYWACQGCVLTGVWVIAHECGHHAFSDYQWLDD 120
V.fordii        TVAFIFYYIATNYFHLLPQPLSYVAWPIYWALQGCVLTGVWVIAHECGHHAFSDYQLLDD 120
G.hirsutum      ILVSIFYYVATTYFHNLPQPLSFVAWPIYWTLQGSVLTGVWVIAHECGHHAFSDYQWIDD 120
S.indicum       VIVFLLYYIATSYFHLLPSPYCYLAWPIYWAVQGCVCTGIWVIAHECGHHAFSDYQWLDD 120
N.tabacum       ILVSVFYYIAITYFHLLPSPYCYLAWPIYWICQGCVCTGIWVIAHECGHHAFSDYQWVDD 120
H.annus         TITAVLYHIATTYFHHLPTPLSSIAWASYWVVQGCVLTGVWVIAHECGHHAFSDYQWVDD 115
A.duranensis    LVAYLLFYIATTYFHKLPYPFSFLAWPIYWAIQGCILTGVWVIAHECGHHAFSKYQLVDD 116
O.europaea      TIASLLCYVASTYIQFLPNPLCYFGWLLYWICQGCVLTGVWVVAHECGHHAFSDYQWLDD 120
S.oleracea      TIAFLLYYVATNYIHLLPKPFNYLAWPVYGFVQGCVLTGVWVIAHECGHHAFSDYQWLDD 119
P.oleracea-1    ILASIIYYLATTYIDLLPKPLSYFSWAVYGFVQGCVLTGLWVIAHECGHHAFSDHQWLDD 119
P.oleracea-2    TIAAILYYLATTYIPLLPKPLNYLAWPIYGYIQGCILTGVWVIAHECGHHAFSDYQWLDD 119

A.thaliana      TVGLIFHSFLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKQKSAIKWYGKYLNNPLGRIM 180
B.nappus        TVGLIFHSFLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKKSDIKWYGKYLNNPLGRTV 180
V.fordii        IVGLVLHSCLLVPYFSWKHSHRRHHSNTASLERDEVFVPKKKSSIRWFSKYLNNPPGRLF 180
G.hirsutum      TVGLILHSSLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKRSSIRWWAKYLNNPPGRFV 180
S.indicum       TVGLILHSALLVPYFSWKYSHRRHHSNTGSLERDEVFVPKPKSRVSWYSKYLNNPLGRVI 180
N.tabacum       TVGLILHSALMVPYFSWKYSHRRHHSNTGSLERDEVFVPKPKSQLGWYSKYLNNPPGRVM 180
H.annus         TVGFVLHSSLLVPYFSWKYSHHRHHSNTGSLERDEVFVPKSRSKVPWYSKYFNNTVGRIV 175
A.duranensis    MVGLTLHSCLLVPYFSWKISHRRHHSNTGSLDRDEVFVPKPKSKVSWYNKYMNNPPGRAI 176
O.europaea      TVGLILHSSLLVPYFSWKYSHRRHHSNTGSLERDEVFVPRVKSGIRWISKNLNNPPGRVL 180
S.oleracea      TVGLVLHSFLLVPYFSWKYSHRRHHSNTGSMEKDEVFVPQRKENMSWFSKYLSNPPGRIL 179
P.oleracea-1    TVGLVLHSFLLVPYFSWKYSHRRHHSNTGSMEKDEVFVPKRKSGLQWFSKYLNNPPGRVL 179
P.oleracea-2    TVGLILHSCLLVPYFSWKYSHRRHHSNTGSMDRDEVFVPKKKDGMKWFSKYLNNPPGRVL 179

A.thaliana      MLTVQFVLGWPLYLAFNVSGRPYDG-FACHFFPNAPIYNDRERLQIYLSDAGILAVCFGL 239
B.nappus        MLTVQFTLGWPLYLAFNVSGRPYDGGFACHFHPNAPIYNDRERLQIYISDAGILAVCYGL 240
V.fordii        TLTITLTLGWPLYLAFNVSGRPYDR-FACHYDPYGPIYTDRERTEIYISDAGVLAVTFGL 239
G.hirsutum      TVTIQLTLGWPLYLAFNVAGRPYEG-LACHYNPYGPIYNDRERLQIYISDVGVLAVTYGL 239
S.indicum       TLVVTLTLGWPLYLLFNVSGRPYNR-FACHFDPYGPIYNDRERLQIFISDAGIIAAVCVL 239
N.tabacum       SLTVTLTLGWPLYLAFNVSGRHYDR-FACHYDPYGPIYNDRERLQIFLSDAGVLGAGYLL 239
H.annus         SMFVTLTLGWPLYLAFNVSGRPYDR-FACHYVPTSPMYNERKRYQIVMSDIGIVITSFIL 234
A.duranensis    SLFITLTLGWPLYLAFNVSGRPYDR-FASHYDPYAPIYSNRERLLIYVSDSSVFAVTYLL 235
O.europaea      LLVFQLTLGWPLYLMFNVSGRHYDR-FACHYNPNSPMYSDRERAQILISDAGVLAVTCGL 239
S.oleracea      TLVVTLTLGWPLYLLFNVSGRKYER-FACHYDPSSPIYSDRERLQIFISDVGISIVAFGL 238
P.oleracea-1    TLTITLTLGWPLYLLFNVSGRHYDR-YACHYDPYGPIYSDRERLQIYISDAGILAVVYGL 238
P.oleracea-2    TLLVTLTLGWPLYLLFNVSGRKYER-FACHYDPYGPIYSDRERLQIYLSDAGILAVSYGL 238
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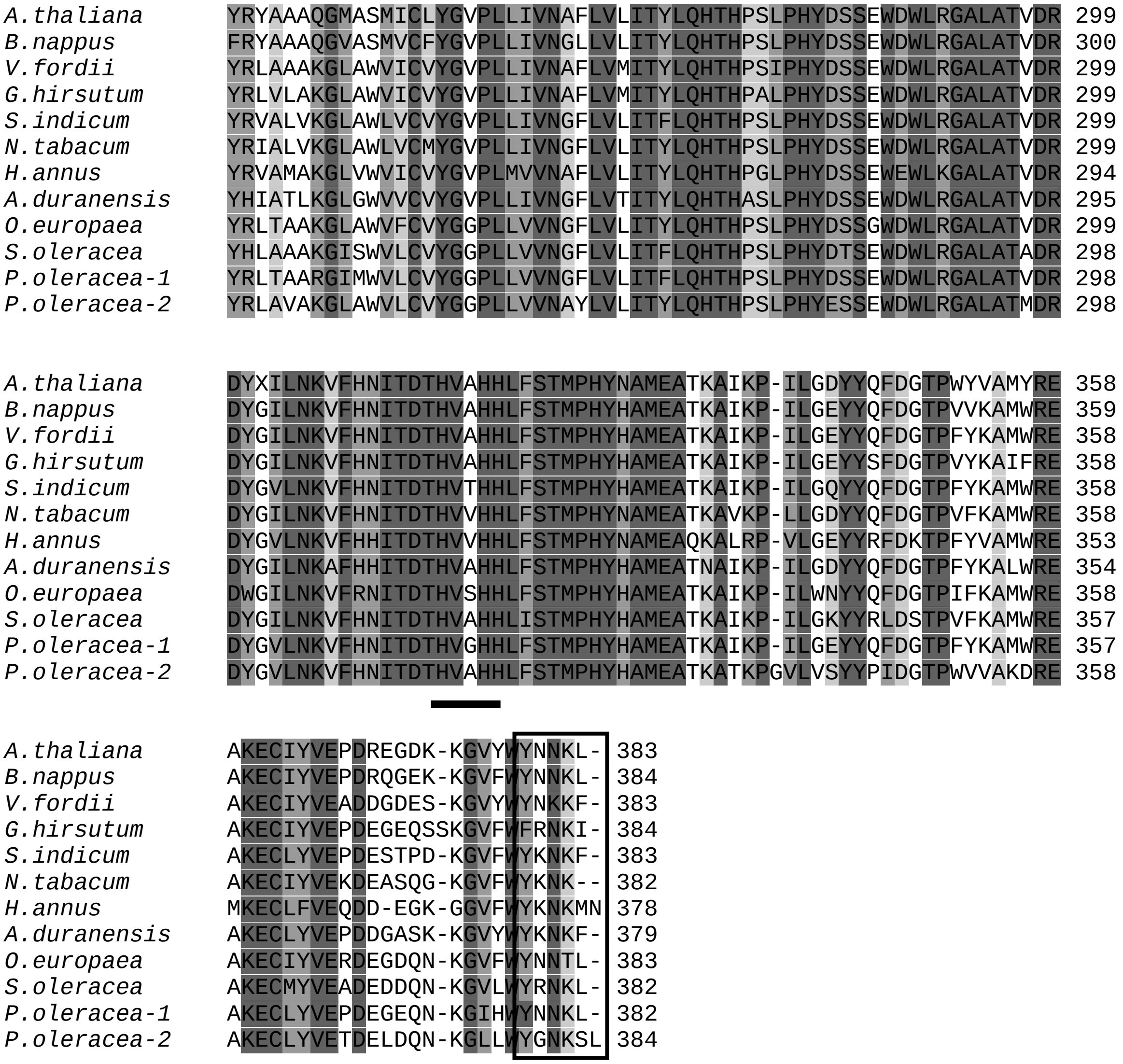

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G.hirsutum      YRLVLAKGLAWVICVYGVPLLIVNAFLVMITYLQHTHPALPHYDSSEWDWLRGALATVDR 299
S.indicum       YRVALVKGLAWLVCVYGVPLLIVNGFLVLITFLQHTHPSLPHYDSSEWDWLRGALATVDR 299
N.tabacum       YRIALVKGLAWLVCMYGVPLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRGALATVDR 299
H.annus         YRVAMAKGLVWVICVYGVPLMVVNAFLVLITYLQHTHPGLPHYDSSEWEWLKGALATVDR 294
A.duranensis    YHIATLKGLGWVVCVYGVPLLIVNGFLVTITYLQHTHASLPHYDSSEWDWLRGALATVDR 295
O.europaea      YRLTAAKGLAWVFCVYGGPLLVVNGFLVLITYLQHTHPSLPHYDSSGWDWLRGALATVDR 299
S.oleracea      YHLAAAKGISWVLCVYGGPLLVVNGFLVLITFLQHTHPSLPHYDTSEWDWLRGALATADR 298
P.oleracea-1    YRLTAARGIMWVLCVYGGPLLVVNGFLVLITFLQHTHPSLPHYDSSEWDWLRGALATVDR 298
P.oleracea-2    YRLAVAKGLAWVLCVYGGPLLVVNAYLVLITYLQHTHPSLPHYESSEWDWLRGALATMDR 298

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B.nappus        DYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKP-ILGEYYQFDGTPVVKAMWRE 359
V.fordii        DYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKP-ILGEYYQFDGTPFYKAMWRE 358
G.hirsutum      DYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKP-ILGEYYSFDGTPVYKAIFRE 358
S.indicum       DYGVLNKVFHNITDTHVTHHLFSTMPHYHAMEATKAIKP-ILGQYYQFDGTPFYKAMWRE 358
N.tabacum       DYGILNKVFHNITDTHVVHHLFSTMPHYNAMEATKAVKP-LLGDYYQFDGTPVFKAMWRE 358
H.annus         DYGVLNKVFHHITDTHVVHHLFSTMPHYNAMEAQKALRP-VLGEYYRFDKTPFYVAMWRE 353
A.duranensis    DYGILNKAFHHITDTHVAHHLFSTMPHYHAMEATNAIKP-ILGDYYQFDGTPFYKALWRE 354
O.europaea      DWGILNKVFRNITDTHVSHHLFSTMPHYHAMEATKAIKP-ILWNYYQFDGTPIFKAMWRE 358
S.oleracea      DYGILNKVFHNITDTHVAHHLISTMPHYHAMEATKAIKP-ILGKYYRLDSTPVFKAMWRE 357
P.oleracea-1    DYGVLNKVFHNITDTHVGHHLFSTMPHYHAMEATKAIKP-ILGEYYQFDGTPFYKAMWRE 357
P.oleracea-2    DYGVLNKVFHNITDTHVAHHLFSTMPHYHAMEATKATKPGVLVSYYPIDGTPWVVAKDRE 358

A.thaliana      AKECIYVEPDREGDK-KGVYWYNNKL- 383
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V.fordii        AKECIYVEADDGDES-KGVYWYNKKF- 383
G.hirsutum      AKECIYVEPDEGEQSSKGVFWFRNKI- 384
S.indicum       AKECLYVEPDESTPD-KGVFWYKNKF- 383
N.tabacum       AKECIYVEKDEASQG-KGVFWYKNK-- 382
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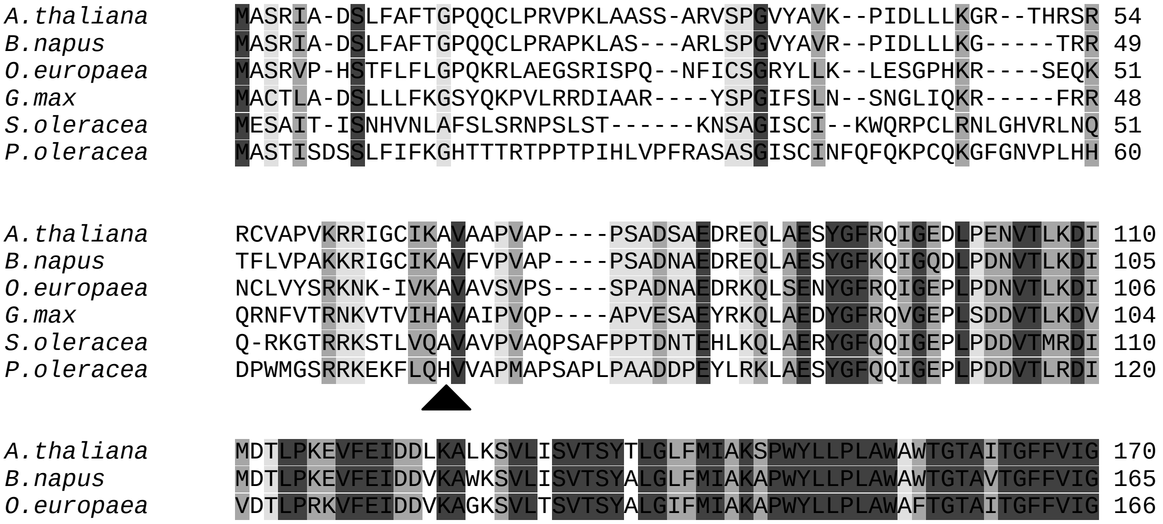
(B)



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O.europaea      MASRVP-HSTFLFLGPQKRLAEGSRISPQ--NFICSGRYLLK--LESGPHKR----SEQK 51
G.max           MACTLA-DSLLLFKGSYQKPVLRRDIAAR----YSPGIFSLN--SNGLIQKR-----FRR 48
S.oleracea      MESAIT-ISNHVNLAFSLSRNPSLST------KNSAGISCI--KWQRPCLRNLGHVRLNQ 51
P.oleracea      MASTISDSSLFIFKGHTTTRTPPTPIHLVPFRASASGISCINFQFQKPCQKGFGNVPLHH 60

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O.europaea      NCLVYSRKNK-IVKAVAVSVPS----SPADNAEDRKQLSENYGFRQIGEPLPDNVTLKDI 106
G.max           QRNFVTRNKVTVIHAVAIPVQP----APVESAEYRKQLAEDYGFRQVGEPLSDDVTLKDV 104
S.oleracea      Q-RKGTRRKSTLVQAVAVPVAQPSAFPPTDNTEHLKQLAERYGFQQIGEPLPDDVTMRDI 110
P.oleracea      DPWMGSRRKEKFLQHVVAPMAPSAPLPAADDPEYLRKLAESYGFQQIGEPLPDDVTLRDI 120

A.thaliana      MDTLPKEVFEIDDLKALKSVLISVTSYTLGLFMIAKSPWYLLPLAWAWTGTAITGFFVIG 170
B.napus         MDTLPKEVFEIDDVKAWKSVLISVTSYALGLFMIAKAPWYLLPLAWAWTGTAVTGFFVIG 165
O.europaea      VDTLPRKVFEIDDVKAGKSVLTSVTSYALGIFMIAKAPWYLLPLAWAFTGTAITGFFVIG 166
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P.oleracea     ISSLPKKVFEIDERKAWGSVLLSVTSYLLGLLMIAKAPWYLLPLAWAWTGTAVTGFFVIG 180

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B.napus        HDCAHKSFSKNKLVEDIVGTLAFLPLVYPYEPWRFKHDRHHAKTNMLVHDTAWQPVPPEE 225
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P.oleracea     HDCAHKSFSRNKLLEDIVGTLAFMPLIYPYEPWRFKHDQHHAKTNMLNEDTAWHPVWKED 240

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O.europaea     FESSPILRKAIIYGYGPFRPLMSIAHWLMWHFDLKKFRSNEVKRVKISLACVSAFIAIGW 286
G.max          FESTPLLRKAIIYGYGPFRCWMSIAHWLMWHFDLKKFRPSEVPRVKISLACVFAFIAIGW 284
S.oleracea     IESSPGLRKALIYAYGPLRTWMSIAHWLKVHFNLKDFRQSEVKRATISLAAVFAFMVIGW 290
P.oleracea     IEASPALRKAMIFGYGPFRTFMSIAHWLLWHFDLKKFRQSEVNRVLISLSAVFAFMIVGW 300

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B.napus        PLIIYKVGVLGWVKFWLMPWLGYHFWMSTFTMVHHTAPHIPFKPADEWNAAQAQLNGTVH 345
O.europaea     PLIILKTGIMGWIKFWLMPWLGYHFWMSTFTMIHHTAPHIPFKSSDEWNAAQAQLNGTVH 346
G.max          PLIIYKTGIMGWIKFWLMPWLGYHFWMSTFTMVHHTAPYIPFKYSEEWNRAQAQLNGTVH 344
S.oleracea     PLIIYKTGIVGWIKFWLMPWLGYHFWMSTFTIVHHTAPHIPFKSSKEWNAAQAQLSGTVH 350
P.oleracea     PLIVYKAGIVGWVKFWLMPWLGYHFWMSTFTIVHHTAPHIPFKQSDKWNAAQAQLNGTVH 360

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B.napus        CDYPSWIEILCHDINVHIPHHISPRIPSYNLRAAHQSIQENWGKYTNLATWNWRLMKTIM 405
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G.max          CDYPKWIEILCHDINVHIPHHISPRIPSYNLRAAHKSLQENWGQYLNEASWNWRLMKTIM 404
S.oleracea     CDYPRWIEILCHDISVHIPHHISPKIPSYNLRAANQSLNENWGEYLNKPKSNWRLMRTIM 410
P.oleracea     CDYPRWIEILCHDINVHIPHHISPRIPWYNLRAAQESIDKNWGKYINKAKWNWRLMRTIM 420

A.thaliana     TVCHVYDK------------------------------ 418
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O.europaea     TICHVYDKEQNYVAFDELAPKDSQPITFLKKVMPDYA- 443
G.max          TVCQVYDKEKSLCCLRRTCP------------------ 424
S.oleracea     TTCHIYDKDGNYVSFEKAVPEESQPISIPKRVMPDYA- 447
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Figure 2

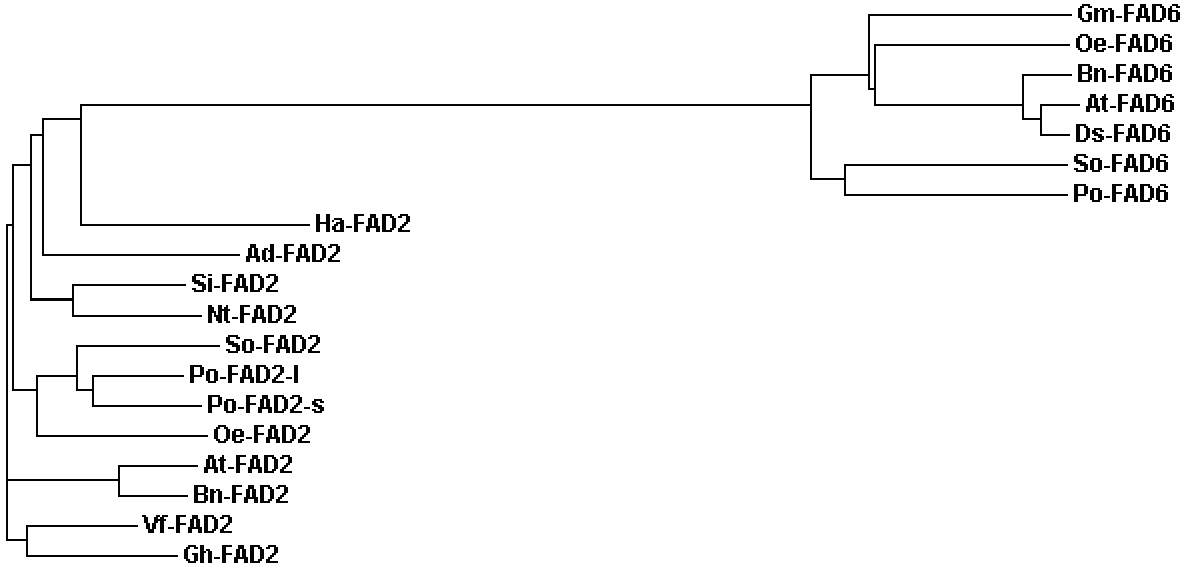


Figure 3

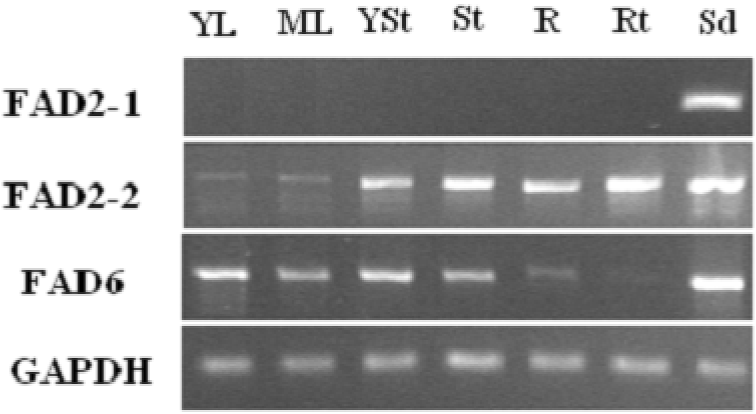


Figure 4

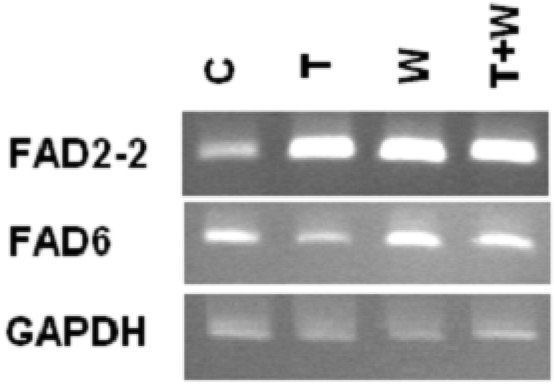
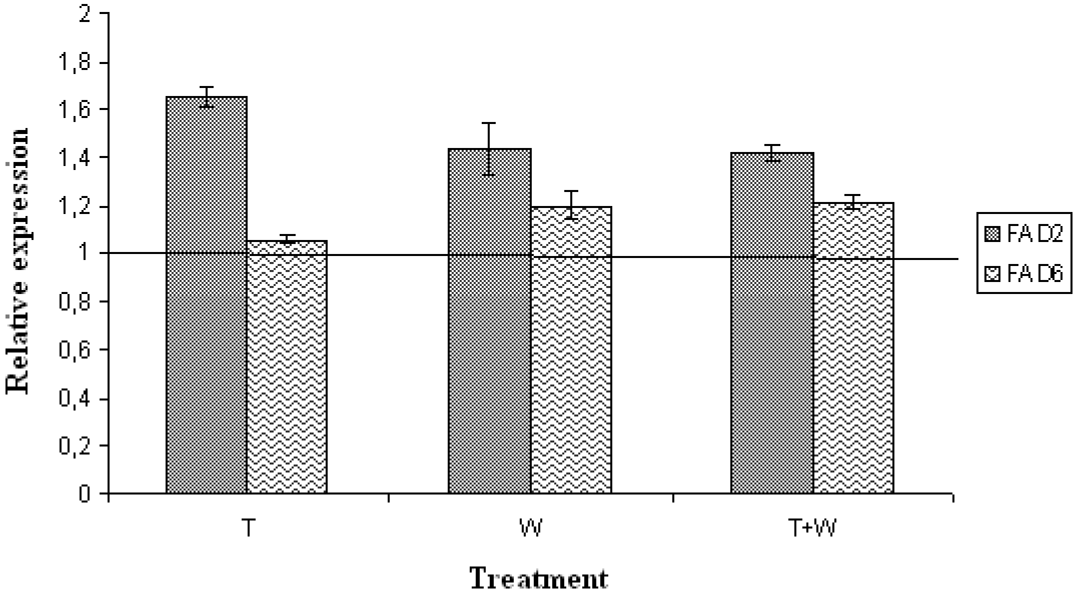


Figure 5



PAPER III

Omega-3 fatty acid desaturase genes isolated from purslane (*Portulaca oleracea* L): Expression in different tissues and response to cold and wound stress.

(Submitted: article ID: jf-2009-009015)

Omega-3 fatty acid desaturase genes isolated from Purslane(Portulaca Oleracea). Expression in different tissues and response to cold and wound stress

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Manuscript ID:	draft
Manuscript Type:	Article
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Complete List of Authors:	Teixeira, Monica; Universidade do Algarve, Biotechnology; IBB/CGB Carvalho, Isabel, S.; Universidade do Algarve, Biotechnology; IBB/CGB Brodelius, Maria; University of Kalmar, school of Pure and Applied Natural Sciences

Omega-3 fatty acid desaturase genes isolated from purslane (*Portulaca oleracea* L):Expression in different tissues and response to cold and wound stress.

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RUNING TITLE: Desaturase genes in response to cold and wound stress

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Abstract:

Two full-length cDNA clones *PoleFAD7* and *PoleFAD8*, encoding plastidial omega-3 fatty acid desaturases were isolated from purslane (*Portulaca oleracea*). The encoded enzymes convert linoleic to α-linolenic acid (C18:3*n*-3). Three histidine clusters characteristic to fatty acid desaturases, a putative chloroplast transit peptide in the N-terminal, and three putative trans-membrane domains were identified in the sequence. Both genes were expressed in all analyzed tissues showing different levels of expression. *PoleFAD7* was up-regulated by wounding, but not by low temperature. *PoleFAD8* was up-regulated by cold stress but not by wounding. Total fatty acid and linolenic acid content were higher both, in wounded and intact leaves of plants exposed to low temperature.

Keywords: *Portulaca oleracea*, omega-3 fatty acid desaturase, *FAD7, FAD8*, Cold stress, Wounding,

List of abbreviations: FAD – Fatty acid desaturase, PUFA – polyunsaturated fatty acid, LA – linoleic acid, ALA -α-linolenic acid, DA – dienoic acid, TA –trienoic acid

INTRODUCTION:

Purslane has a long history of use for human food, animal feed and medicinal proposes. However these days, most people in modern societies limit their diet to a few cultivated vegetables so that wild plants such as purslane tend to be under-utilized. A nutritive characterization of purslane accessions conducted by Ezekwe *et al.* (1999) showed that, in spite of its genetic diversity, purslane remains one of the most abundant terrestrial vegetable sources of omega-3 fatty acids and other essential nutrients potentially beneficial for humans and animals. The presence and concentration of α-linolenic acid in purslane may vary with the cultivar, geographic distribution, developmental stage and environmental factors [1, 2, 3, 4]. Total fatty acid content differs among purslane tissues. It was found to be higher in seeds and leaves [2, 5, 6], and lower in stems (about one third). Linolenic acid (ALA) accounted for 60% of TFA in leaves, 30-40% in seeds and 10 to 25% in stems. Except for linseed, in which ALA is 50-60% of the total fatty acids, most other oil crop seeds contain only 3 to 10 % ALA. So, it is reasonable to say that purslane is an excellent vegetable source of ALA.

In plants, dienoic (DA) and trienoic (TA) acids usually account for as much as 70 % of total lipids. Oil producing crop plants synthesize and storage energy in the form of triacylglycerols, which are composed of a glycerol backbone molecule esterified by three saturated and/or unsaturated fatty acyl groups [7]. Unsaturated fatty acids are essential components required for normal cellular function, being involved in roles ranging from membrane fluidity to acting as signal molecules [8, 9, 10]. Trienoic acids also serve as precursors for several fatty acid-derivate signal molecules, such as traumatic acid and jasmonic acid [11]. In particular, the class of fatty acids known as the polyunsaturated fatty acid*s* (PUFAs) has attracted considerable interest as pharmaceutical and nutraceutical compounds [9, 12]. PUFAs can be defined as fatty acids of 18 carbons or more in length, containing two or more double bonds. These double bonds are inserted by specific fatty acid desaturase enzymes that have been the subject of intense research in recent years [13, 14]. Linoleic and α-linolenic acids are essential fatty acids synthesized in plant tissues from oleic acid by the introduction of double bonds between the existing double bond and the terminal methyl group by the sequential action of Δ12 and Δ15 desaturases. Omega-3 fatty acid desaturases catalyzes the insertion of a third double bound into linoleic acid precursors to produce linolenic acid. Three distinct desaturases have been characterized that can insert the Δ15 double bond, i.e. two ω-3 desaturase plastidial enzyme (*FAD7 and FAD8*), which uses the terminal methyl group as a reference point, and secondly an extra-plastidial oleate Δ15 desaturase (*FAD3*) [7]. Many omega-3 FAD genes have been isolated and characterized from a diverse pool of plant species. Genes encoding two plastidial omega-3 desaturases (*FAD7*/*FAD8*) were isolated from *Glycine max* [15], *Arabidopsis thaliana* [16, 17], and *Zea maize* [18]. Here we report on the cloning and expression analysis of two purslane (*Portulaca oleracea* L.) cDNAs encoding two plastidial ω-3 desaturases, *PoleFAD7* and *PoleFAD8.*

Fatty acid desaturases in all organisms are subjected to several types of regulation, depending on their localization or function, and there may be requirement for different or specific desaturase activities during certain development processes in various tissues [19]. Therefore, in order to elucidate expression patterns of these desaturases in purslane, transcript accumulation levels of these genes were investigated in different tissues of purslane plant.

Plants often encounter the abiotic stresses of low or elevated temperature, exposure to salt, drought, and, less commonly, heavy metals, as well as biotic pathogen and insect attack, sometimes simultaneously. Even with the best available land and agricultural practices, the impacts of these stresses can significantly reduce the productivity of food and fiber crops [20].

Thus, the current situation of diminishing farm land worldwide and the potential heightened effects of global climate change on environmental, pathogen and insect stresses provide increased impetus to understand stress resistance in crop plants. More complete knowledge of fatty acid unsaturation, mobilization, and regulation processes may significantly aid the development of effective strategies for managing abiotic and biotic stresses for these plants [20]. Fatty acid desaturases (FAD), such as oleic and linoleic desaturases, play multiple roles in plants. FAD can modulate the membrane fluidity by modifying the degree of lipid unsaturation to respond to temperature changes. Experiments using seed and leaf tissues of soybean and wheat showed that the levels of α-linolenic ($C18{:}3n$-3) and linoleic ($C18{:}2n$-2) gradually increased as the temperature decreased [21, 22].

In order to elucidate the effect of chilling and wounding stresses on the expression patterns of *FAD7* and *FAD8* genes in purslane leaves, transcript accumulation levels of these genes and linolenic acid accumulation were investigated in intact and wounded leaves of purslane plants exposed to standard (25 ºC) and low (5 ºC) growth temperatures.

MATERIAL AND METHODS

Plant materials

Young expanding leaves, mature leaves, young stems, mature stems, shoot tips, roots and seeds were harvested from 1-month-old seedlings of purslane (*Portulaca oleracea* L.), grown in a growth chamber at 23 ºC under 16-h photoperiod. All samples were immediately frozen in liquid nitrogen and stored at -80 ºC until further analysis. For expression analysis under cold stress, 1 month-old *P. oleracea* plants were placed at 5 ºC for 24h. For expression analysis under wounding stress, two 1 cm-long fissures were performed into leaves of a 1-month-old *P. oleracea* plants across the midrib with a sterile razor blade, and then incubated for 24 hours under normal and cold growth conditions. All samples, taken from wounded or intact leaves of control or cold-stressed plants, were immediately frozen in liquid nitrogen and stored at -80 ºC until further analysis. These samples were used for total RNA extraction and Fatty acid analysis in a GC-MS system.

2.2. PCR amplification and cloning of two omega-3 FADs cDNA Fragments

Primers to amplify cDNA fragments corresponding to plastidial omega-3 desaturase genes in *Portulaca oleracea* were designed based on conservative nucleotide sequences of these homologous genes from *Arabidopsis thaliana* and other orthologues. The primers: *FAD3*-1fw (5'-CTC/TGGG/A/C/TCAC/TGATCTGC/TGGG/A/C/TCATGG-3') and *FAD3*-1rv (5'-TGA/GTAA/GTGA/C/TGGA/GATC/TTGG/A/C/TGGA/GAA-3') were used to amplify the central region of *PoleFAD7* and *PoleFAD8* clones. Total RNA was isolated from different *P. oleracea* tissues using a Plant RNA Purification Reagent Kit (Invitrogen, Carlsbad CA, USA). Using the RT-PCR system (Fermentas), the first strand cDNA was synthesized with 2 µg purified total RNA (pre-treated with DNase I) according to the manufacture's protocol. The oligo(dT)$_{18}$ was used as a primer. PCR was performed in a 25 µl reaction mixture using PuReTaqTM Ready-To-Go PCR beads (PuReTaq Polymerase (2.5 units), GE Healthcare) together with 1 µl of 10 mM of each primer and 2 µl of RT-products. The reaction mixture was subjected to an initial denaturation for 3 min at 94ºC, and subsequently 30 cycles of 30 s at 94ºC, 30 s at 55ºC, and 90s at 72ºC followed by one cycle of 7 min at 72ºC. The PCR products were run on 1% agarose gel and the target bands were purified cloned into pGEM-t easy vector (Promega) and sequenced (Eurofins, Germany).

2.3. Rapid amplification of cDNA ends (RACE)

For amplification of unknown 3’- and 5’-ends of the above conservative cDNA fragments, the 3’- and 5’-RACE were performed according to protocols described in the GeneRacer Kit (Invitrogen, USA). Based on sequence information of the conservative cDNA fragments, gene specific primers (GSP) were designed, respectively 5’*FAD7* rv1 (5’-GACCAACAAGCTGTGGAAGTTA-3’), 5’*FAD7* rv2 (5’-GGGTTGAATAAATCACTGTCTGGGTGATA-3’), 5’*FAD7* rv3 (5’-GCCTGACTTTCCTGGACTTCTTTTCCACAA-3’), 5’*FAD7* rv4 (5’-AAACCCCTCCGGCGACCCATTGACC-3’) 5’*FAD7* rv5 (5’-AATCCTCCGCCGCCGTAGACGGC-3’) 5’*FAD7* rv6 (5’- AATGGGGCGGCGACATTCACCTCCC-3’) for *PoleFAD7*, and *FAD8_3R* GSP (5’- CCACGACATCGGAACTCACGTCATT-3’), 5’*FAD8* rv1 (5’-CCTTGTGGTTTCAGGCACACTTCTAT-3’), 5’*FAD8* rv2 (5’-GGCCACGGATGCCATGACTCGTCGTT-3’), 5’*FAD8* rv3 (5’- CCATGGTTTGCATGATGAGTCCTGTGACTTA-3’), 5’*FAD8* rv4 (5’-GCAGCCATGGCCATGAATGCAGCAA-3’), 5’*FAD8* rv5 (5’-GGTGGTGGTGCCCCATGATCAAACCCTAAT-3’), 5’*FAD8* rv6 (5’- GGTGAGCATTTGTGGGGTGCTAGGGTTA-3’), for *PoleFAD8*, respectively. To amplify the full-length *PoleFAD7* and *PoleFAD8* cDNAs, primers were designed based on the assembled sequences, respectively: *FAD7*flfw (5’- ATAGGATCCGGAATGGCGAGTTGGGTACTCTC-3’)/*FAD7* flrv (5’-TGCGAATTCCAAAAGCTCAGGGTCTG-3’); *FAD8* flfw (5’-ATAGGATCCGGAATGGCGAGTTGGGTACTCTCA -3’)/*FAD8* flrv (5’-TCATGTTCTTTGTCCATTGAGTTTTGGAT-3’).

2.4. Semi-quantitative RT-PCR assay

The steady state levels of *PoleFAD7* and *PoleFAD8* transcripts were estimated in various purslane tissues and in seed. The FAD2 RT-PCR fw (sense) and FAD2 RT-PCRrv (antisense) primers were used for real-time semi-quantitative PCR analysis of the *PoleFAD2* gene, designated as mentioned above, and for the *PoleFAD6* gene the FAD6 RT-PCR fw (sense) and FAD6 RT-PCRrv (antisense) primers, both designed from the coding region of the correspondent genes. We tested three control genes for transcript analysis. Elongation factor 1 (EF1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquinin (UBI). We determined that GAPDH should be used as control for spatial expression of the genes in purslane tissues since it showed steady state expression in all analyzed tissues. UBI was used as control in the stress assay becouse GAPDH showed some expression variation in the stress samples. So a part of the coding region of GAPDH and UBI genes were amplified with the specific pairs of primers GAPDHfw (5'-CACGGCCACTGGAAGCA-3')/GAPDHrv (5'-TCCTCAGGGTTCCTGATGCC-3'), and UBIfw (5'-GGCATGCAGATCTTTGTGAAGAC-3') and UBIrv(5'-GGGATACCCTCCTTGTCCTG-3'), respectively. PCRs were performed using equal amounts of templates (25 ng cDNA) and gene specific or GAPDH primers and carried out for different number of cycles in order to optimize reproducibility and ensure that reactions remained in the log-linear range. Relative expression of target genes in purslane tissues was calculated using the efficiency calibrated Model [23]. In this model, for each sample, target gene and a reference gene for internal control are included in the PCR amplification from serially diluted samples. The relative expression is given by a ratio between the target gene and the reference gene, calculated according to equation 1.

$$\text{Relative expression (RE)} = (E_{\text{target}})^{\Delta\text{Ct target}} / (E_{\text{reference}})^{\Delta\text{Ct reference}} \qquad \text{Equation 1 [23]}$$

Assays of transcript levels for each target gene in the stress assay were normalized by Ubiquinin (UBI) gene expression for ΔCt value calculation. Target gene expression level in plants exposed to the treatments was also normalized by the expression level of each corresponding gene in the leaves of control plants. We considered an up-regulation of the gene for RE ≥ 1.5 and a down regulation for RE ≤ 0.5.The RT-qPCR protocol was optimized by determining the optimal primer concentration and primer efficiency. The RT-qPCR products were separated by 1% agarose gel electrophoresis. The RT-qPCR fragments were cloned in *E. coli* and sequenced by Eurofins MWG GmbH (Germany), confirming the identity of the amplified products. .

2.5. Sequence analysis

Nucleotide sequences from cDNA clones and deduced amino acid sequences were identified by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Predictions of open reading frames (ORFs) were made using the DNALC Bioinformatics software (http://www.dnalc.org/bioinformatics/2003/2003_dnalc_nucleotide_analyzer.htm). Theoretical molecular weights of deduced polypeptides and hydropathy analysis were made by using the Peptide properties calculator software (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp) and transmembrane regions were predicted by the HMMTOP server ver.2.0 (http://www.enzim.hu/hmmtop/html/submit.html). Predictions of subcellular localization of the deduced polypeptides were conducted by using PSORT (http://www.psort.nibb.ac.jp./form.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) algorithms. Amino acid multiple alignments were made with the ClustalW program under default parameters. A phylogenetic tree was constructed using the neighbor-joining algorithm included in the ClustalW program. The tree was visualized using the TreeView program.

2.6. Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared using 3N methanolic-HCL as described by Vriten *et al*. (2005) [24]. FAME were analyzed on a Hewlett Packard 5890 series II gas chromatography (GC) instrument equipped with a SGE 25QC2/BPX-70 (25m x 220µm x 0.30 µm) Supelco column, connected to a mass detector Agilent 5973 Network. The samples were injected with an automatic injector Agilent 7683 Series in the split-less mode at 250 ºC. The oven was programmed to hold temperature at 60 ºC for 2 min, increase to 170 ºC at 30 ºC/min and hold 5 min, increase to 200 ºC at 4 ºC/min and hold 5 min, increase to 250 ºC at 50 ºC/min and hold for 5 min. Helium was used as a carrier gas. Temperatures of ionization source and quadruple were 230 ºC and 150 ºC , respectively. FAMEs were identified by comparative analysis of retention times to standards and % FAME was calculated based on peak area counts, in relation to a internal control (heneicosanoic acid C21:0). All lipid analysis experiments were performed independently at least three times. Values are reported as average and standard deviation.

RESULTS

Cloning and sequence analysis of purslane ω-3 desaturases full-length cDNAs

Based on the nucleotide sequences of *FAD7* and *FAD8* from different plant species, two pairs of primers were designed, respectively. Using these primer pairs, two conserved cDNA fragments were obtained by PCR using cDNA aliquots of *P. oleracea* L. leaves. To clone the full-length cDNA, 3'-and 5'-RACE reactions were conducted. The 3'-RACE-PCRs, generated a fragment of 481 bp for the *PoleFAD7* and 493 for *PoleFAD8* gene. The 5`RACE reactions generated fragments of 395 bp and 500 bp, for *PoleFAD7* and *PoleFAD8*, respectively. Sequence comparisons of the 3'- and 5'-ends with the conservative fragments of these genes previously amplified showed overlapping regions that matched perfectly. Blast searches of the deduced amino acid sequences revealed that these sequences represented the missing parts of *PoleFAD7* and *PoleFAD8* genes. Based on the above sequences data, primers were designed for the 3'- and 5'-UTRs of these genes, and the full-length cDNA was amplified, cloned and sequenced, respectively.

Descriptions of the properties of the two distinct *P. oleracea* cDNAs are listed in Table 1. Inspection of both sequences revealed the presence of eight highly conserved histidine residues in three separate clusters corresponding to the amino acid residues 169-173, 205-209, 372-376, for PoleFAD7, and residues 172-176, 208-212, 376-380 for *PoleFAD8*. The residues are necessary for the function of all membrane-bound fatty acid desaturases [13]. These invariant residues are arranged in three histidine boxes (HXXXH, HXXHH, and HXXHH) with conserved spaces between them (Fig. 1) these histidine boxes participate in the formation of active sites with iron and are conserved in all omega-3 type FA desaturases [10]. The putative chloroplast transit peptide sequence has, respectively, 52 and 74 amino acids at the N-termini of *PoleFAD7* and *PoleFAD8* (Fig. 1). The N-terminal sequences also had several characteristics of plastidial transit peptides, including a high content of hydroxilated residues (Ser, Thr), a low content of acidic residues and the conserved N-terminal Met-Ala dipeptide [25].

Multiple sequence alignment revealed high similarities of the two distinct omega-3 FAD amino acid sequences to other orthologous desaturase sequences available in the GenBank.

PoleFAD7 and *PoleFAD8* exhibited 68-76 % and 60-67 % of identity to other *FAD7* and *FAD8* proteins from different plant species, respectively. The sequence identity between *PoleFAD7* and *PoleFAD8* was 63 %.

Hydropathy analysis of *PoleFAD7* showed that the encoded protein contained three putative membrane spanning domains at amino acid residues, 133-152, 283-305 and 312-330. *PoleFAD8* also contained three putative membrane-spanning domains at amino acid residues 134-153, 284-306, and 313-331. Three to four putative trans-membrane domains were also detected in *FAD7* and *FAD8* translated sequences of other plant ortologues such as *A. thaliana*, *S. indicum* and *B. napus*, in similar positions. The prediction of three putative trans-membrane regions for *PoleFAD7* and *PoleFAD8* polypeptides supported the notion that the two desaturases are membrane–bound.

The neighbor-joining phylogenic tree (Fig.2) showed that *PoleFAD7* and *PoleFAD8* are distributed in the same group, which indicate that they share common characteristics, like sub cellular location, corroborated by the presence of a chloroplastidial signal peptide in the N-terminal on both gene sequences. We can also see that *PoleFAD8* sequence is very divergent in relation to *PoleFAD7* and other plastidial *FAD*, being placed in an isolated branch, closer to *A. thaliana* and *D. sophia FAD7* and *FAD8* genes. *PoleFAD7* showed a closer relation to *S. indicum FAD7*.

Spatial analysis of *Portulaca FAD7* and *FAD8* expression in purslane tissues

There were similar expression profiles among the two distinct omega-3 desaturases in *P. oleracea* tissues (Fig.3). A dominant but not restricted expression of *PoleFAD7* was observed in photosynthetic tissues particularly in the young developing leaves and stems. Expression of *PoleFAD7* was also detected in root and seeds. A low expression level of *PoleFAD8* gene was detected in all purslane tissues, with slight higher levels in stems and root tips (Fig. 3). In *A. thaliana* this gene has been characterized as temperature-dependent, being expressed when the plants are exposed to low temperatures and undetectable at optimal growth temperature [16]. If PoleFAD8 share the same characteristic, it could explain the low but detectable levels of expression observed, since the plants were grown at 25ºC, five degrees below the optimal growth temperature.

3.3. Effect of chilling and wounding on linolenic acid accumulation and omega-3 desaturase gene expression on purslane leaves

The chloroplast membranes of higher plants have unusually high concentrations of trienoic fatty acids, with linolenic (C18:3) or a combination of linolenic and hexadecatrienaic (C16:3) acids, making up more than 80% of the fatty acids found in this organelle [7, 26]. Although the processes of acclimatization is considered to include complex and various biochemical processes, Kodama *et al.* (2005) shown that an increase in total fatty acid content, during the acclimation process was one of the important factors in chilling tolerance in tobacco leaves [27]. An increase in trienoic acids, particularly C16:3, was also detected in leaves of plants grown at low temperature (15 ºC). In our work we also detected an increase in total fatty acid content in purslane leaves of chilled and wounded plants, particularly the linoleic (C18:2) and linolenic (C18:3) acids content (Table 2). The increase in the insaturation level in the membrane may contribute to plant survival at low temperatures in purslane plants. We also detected an increase on linolenic acid accumulation in wounded purslane leaves, which may be associated to the activation of octadecanoic pathway to produce JA, since linolenic acid is a known precursor for this stress-signaling molecule. The higher level of linolenic acid was detected in leaves of plants subjected to both, low temperature and wounding, but no cumulative effect was noticed (Table 2).

To determine *PoleFAD7* or *PoleFAD8* genes transcript level in leaves of purslane plants submitted to chilling and wounding stresses, semi-quantitative real-time PCR analysis was carried out. cDNA was synthesized from total RNA extracted from intact and wounded leaves of plants grown at 25 ºC and plants exposed to chilling temperature, 5 ºC, for 24 hours. In purslane plants grown at 25ºC, *PoleFAD7* and *PoleFAD8* transcripts were detected in intact leaves, although *PoleFAD8* was expressed in very low level (Fig.4A). In response to low temperature, a slight decrease in expression level was detected for *PoleFAD7* gene. On the other hand, *PoleFAD8* transcripts level increase was observed after exposure to 5ºC (Figs. 4A and B) and became dominantly expressed in relation to *PoleFAD7*. A 3.5-fold increase in the *PoleFAD8* expression level was detected in leaves of plants exposed to chilling temperatures (Fig .4B). With respect to wounding, *PoleFAD7* was dominantly expressed, although a slight increase in *PoleFAD8* was also present (Figs. 4A). No cumulative effect was noticeable when the two treatments, low temperature and wounding, were applied simultaneously, but 3-fold increase on *PoleFAD8* was detected (Fig.4B).

DISCUSSION

Fatty acid desaturases have a key role of for the production of polyunsaturated fatty acids, which have several important roles in both plant development and defense response [28, 29], and alternatively possess crucial role to polyunsaturated acid content of vegetable oils, which is important factor for oil quality.

In the plant fatty acid desaturation pathway, the first step is catalyzed by stearoyl-ACP desaturase to convert stearic acid into oleic acid. Plant microsomal and plastidial ω-6 fatty acid desturases, *FAD2* and *FAD6* in Arabidopsis, are the two enzymes responsible for converting oleic acid to linoleic acid through the eukaryotic and the prokaryotic pathway, respectively. Further desaturation of linoleic acid to produce trienoic fatty acids is catalyzed by ω-3 fatty acid desaturases. One microsomal (*FAD3*) and two plastidial (*FAD7* and *FAD8*) omega-3 fatty acid desaturases are responsible for the conversion of linoleic into linolenic acid [7].

In the present study, using a PCR-based approach, we have cloned two distinct plastidial ω-3 fatty acid desaturase cDNAs from purslane, designated as *PoleFAD7* and *PoleFAD8*. Based on multiple alignments of their deduced amino acid sequences and phylogenetic analysis it was shown that both genes, *PoleFAD7* and *PoleFAD8* had a relatively high homology to other plant ω-3 fatty acid desaturases that have putative plastidial transit peptides. The *FAD7* and *FAD8* genes of *Portulaca oleracea* L. have been isolated and characterized by genetic criteria as encoding chloroplast-localized omega-3 desaturases.

Expression pattern of these two genes in purslane tissues was analyzed by real time PCR analysis, and we detected similar expression profiles among the two distinct omega-3 desaturases (Fig.3). *PoleFAD7* showed a dominant, but not restricted, expression in photosynthetic tissues, such as leaves and stems (Fig. 3), being also detected in root and seeds. The transcript of the *FAD7* gene was observed only in the chlorophyllous tissue of *Arabidopsis* [30] and tobacco [31] plants, however other reports have indicated that *FAD7* gene expression can also be detected in non-photosynthetic tissues, including developing embryos of linseed [32], in maturing pollen grains of *B. napus* [33], and in the flower apparatus of *O. europaea* [34].

Expression of *PoleFAD8* gene was detected in all organs, although at low levels, with slight higher levels in stems and root tips (Fig. 3). Purslane is a semi-tropical plant, with an optimal growth temperature of 30ºC, so growing the plant at 25 ºC may have activated the transcription of *PoleFAD8*, leading to detectable levels of accumulation in all analyzed tissues. In *Descuraina sophia* plants, *FAD8* gene expression was restricted to photosynthetic tissues such as leaves, stems and young siliques [35].Much of the interest in fatty acid desaturation is the result of a proposed correlation between the high degree of membrane lipid polyunsaturation and tolerance to low and freezing temperatures.

The deleterious effects of low temperature on mutants of Arabidopsis with reduced levels of polyunsaturation are direct evidence that fatty acid composition is a component of low temperature fitness [36, 37]. The importance of trienoic fatty acid composition in conditioning cold tolerance was also demonstrated in transgenic tobacco plants that have increased levels of trienoic acids [38]. We also detected an increase in total fatty acid content, particularly linolenic acid, in wounded and intact leaves of chilled purslane plants (Table 2). A cumulative effect of the two treatments in the total fatty acid content or linolenic acid content was not detected, but higher values were obtain when both treatments were applied simultaneously (Table 2).

The effect of chilling and wounding in the transcript level of omega-3 desaturase genes, *PoleFAD7* and *PoleFAD8* was also analyzed. Total RNA isolated from purslane plants grown at 25 ºC contained detectable levels of *FAD7* and *FAD8* genes. A slight reduction of *Portulaca FAD7* transcript accumulation level was detected in the plants exposed to chilling temperatures, which may indicate that this gene plays a negative role in the cold-stress response (Fig. 4B).

However, *FAD8* mRNA that was detectable by real-time PCR in control plants, showed an increased in transcript levels in plants exposed to chilling temperatures (5 ºC) for 24 hours, becoming dominantly expressed in relation to *PoleFAD7*. These differences in steady-state mRNA levels could be the result of alterations either in the rate of *FAD8* transcription or in the stability of the *FAD8* mRNA. A similar result was reported by Tang *et al.* (2007), who detected a down-regulation of *Descuraina Sophia FAD3* and *FAD7* genes in leaves induced by cold stress, together with an up-regulation of *FAD8* gene [35]. Gibson *et al.* (1994) also reported an increased in *FAD8* mRNA transcript level in Arabidopsis plants grown at low temperature (20 ºC), compared to plants grown at 30 ºC. It was also stated that the enhanced expression of Arabidopsis *FAD8* gene at low temperatures indicate the existence of a mechanism that responds to temperature rather to the physical characteristics of the membrane [16]. Therefore, the function of the *Portulaca FAD8* desaturase might be to provide a rapid increase in chloroplast omega-3 desaturase activity after a sudden cold exposure, particularly in rapid growing tissues where rates of fatty acid desaturation may not mach rates of fatty acid synthesis.

Plants respond to wounding by activating a set of defensive genes, such as proteinase inibitor II (*pinII*), and most of these play some role in wound healing, and the prevention of subsequent pathogen invasion [20, 39]. Membrane-associated omega-3 acid desaturase catalyze the desaturation of dienoic acids (C16:2 + C18:2) to trienoic acids (C16:3 + C18:3), which is the starting material for the biosynthesis of fatty acid-derivate signaling molecules such as JA [9, 39, 40, 41]. Jasmonic acid (JA), a fatty-acid derivate hormone, is one of several candidate molecules for wound signaling, and is thought to play a pivotal role in the transcriptional activation of wound-inducible genes [9, 39, 42]. Wounding activates the octadecanoic pathway in which linolenic acid is converted to JA, resulting in a significant accumulation of this hormone.

We observed an up-regulation of purslane *FAD7* gene in wounded leaves; this is consistent with findings in tobacco, Arabidopsis and *Descurainia sophia* where omega-3 FAD genes expression was up-regulated after wounding [30, 31, 35]. Wounding treatments increased the amount of *FAD7* mRNA but not the amount of *FAD3* mRNA in tobacco [31]. The *FAD7* gene was similarly activated in leaves and roots of *Arabidopsis* [30]. Thus is likely that *FAD7* genes play a role in the wound response of higher plants by supplying a precursor for JA biosynthesis [43]. We did not observe a significant increase in *Portulaca FAD8* gene expression when only wounding stress was applied. It is possible that this gene is regulated by a temperature-responsive transcriptional activation, rather than a wound-responsive one in purslane. Post-translational regulation of *Portulaca FAD7* and *FAD8* genes can also play a role in the increase of trienoic acid accumulation on the membranes associated to both, low temperature and wounding stresses. The overexpression of these genes in this plant may play a role in purslane resistance to stresses such as temperature and mechanical injury, by increasing the insaturation level of the cell membrane lipids. This raises the possibility of using these genes, by recombinant technology, to improve resistance to stress in economical important crops such as tomato and potato. The precise molecular mechanism by which this regulation takes place remains unclear and needs further experimental research.

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Figure & Table Legends

Table 1. Description of three full-length cDNAs encoding plastidial omega-3 fatty acid desaturases from *Portulaca oleracea* L.

Table 2. Linoleic (C18:2) and linolenic (C18:3) content in purslane leaves of plants exposed to chilling and/or wounding stresses.

Fig. 1. (A) Alignment of the deduced amino acid sequences determined for *PoleFAD7* (ABL61262) and previously published plastidial ω-3 fatty acid desaturases from *Arabidopsis thaliana* (BAA03106), Brassica juncea (CAB85467), *Sesamum indicum* (AAA70334), *Helianthus annuus* (AAP78965), *Zea mays* (BAA22441), *Crespis alpina* (ABA55807), *Brassica napus* (AAA61774), *Glycine soja* (AAA61776) , and *Descurainia sophia* (ABK91880). (B) Alignment of the deduced amino acid sequences determined for *PoleFAD8* (EU872255) and previously published plastidial ω-3 fatty acid desaturases from *Arabidopsis thaliana* (AAA65621), *Descurainia sophia* (ABK91881), *Vigna unguiculata* (ABY60737), and *Oryza sativa* japonica (AAW32557). The arrowed indicates a putative signal peptide cleavage site. Numbers in parentheses refer to GenBank accession numbers. Identical or similar amino acids are shaded black or grey, respectively. The three characteristic histidine boxes encountered in *FAD7* or *FAD8* subfamilies are underlined. Numbers to the right indicate the position of amino acids.

Fig. 2. Phylogenetic relationships between deduced amino acid sequences from *PoleFAD7* and *PoleFAD8* cDNAs and other plant microsomal plastidial oleate desaturases. At, *Arabidopsis*

thaliana; Bn, *Brasica napus*; Bj, *Brassica juncea*; Os, Oryza sativa japonica; Ds, *Descurainia Sophia*; Ha, H*elianthus. annuus*; Si, *Sesamum indicum*; ; Vu, *Vigna unguiculata*, Ca, *Crespis alpina; Gs,* Po, *Portulaca oleracea*. The GenBank accession numbers of the above sequences are shown in Fig. 1. The tree was constructed by using the Neighbor-Joining algorithm.

Fig. 3. Real-time PCR spatial analysis of *PoleFAD7* and *PoleFAD8* genes. First strand cDNAs were synthesized from total RNA extracted from young expanding leaves (YL), full-expanded mature leaves (ML), young stems tips (YSt), stems (St), roots (R), root tips (Rt) and seeds (Sd). To ensure equal amounts of template, GAPDH was used as a reference gene.

Fig. 4. Real-time PCR analysis of *PoleFAD7* and *PoleFAD8* genes expression on leaves of stressed purslane plants. First strand cDNAs were synthesized from total RNA extracted from intact (C) and wounded (W) leaves of plants grown at 25 ºC and plants exposed to chilling temperature, 5 ºC, for 24 h (T and T+W, respectively). (A) Agarose gel image of *PoleFAD7* and *PoleFAD8* genes expression pattern under stress conditions. (B) Relative expression of target genes in leaves of *P. oleracea* plants subjected to chiling and wounding treatments. Assays of transcript levels for each target gene were normalized by Ubiquinin (UBI) gene expression for ΔCt value calculation. Target gene expression level in plants exposed to the treatments was also normalized by the expression level of each corresponding gene in the leaves of control plants. The continuous line corresponds to a relative expression level of 1, in which the target gene expression from the plant exposed to the wounding and chilling treatments equals the target gene expression in the control plants. The interupted lines correspond to the upper and lower limits considered for a up- or down-regulation of the gene.

Table 1.

Name	Full-length cDNA (bp)	ORF (bp)	Encoded amino acid	Protein molecular mass (KDa)	Isoelectric point	Predicted subcellular location
PoleFAD7	1523	1338	446	50.5	7.5	Chloroplast
PoleFAD8	1606	1362	453	52.4	8.2	Chloroplast

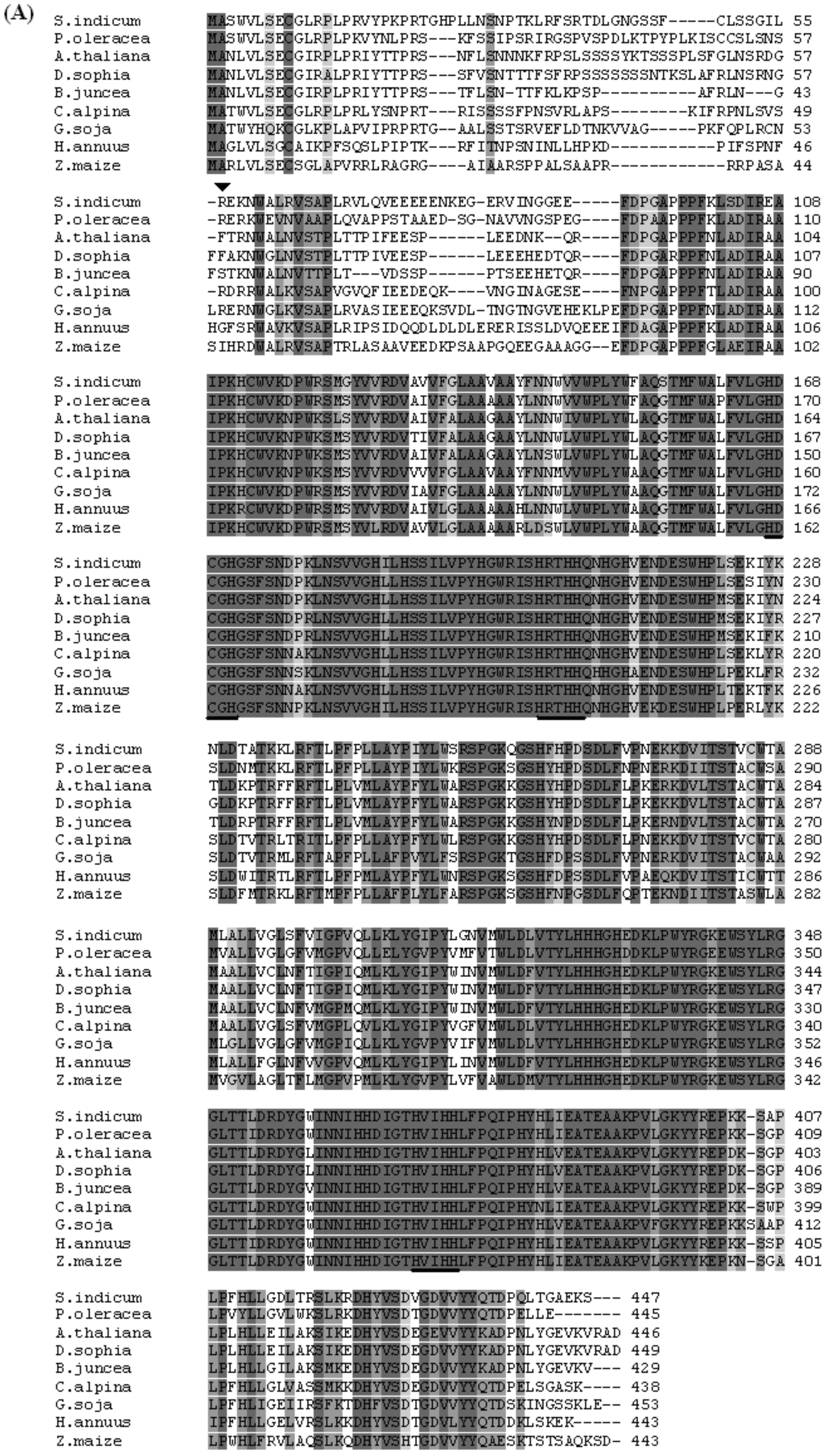
Table 2.

Treatment	Total FA (mg/g leaf FW)	PUFA (mg FA/g leaf FW)		
		C18:2	C18:3	C18:3/C18:2
C	3.41 (0.29)	0.513 (0.08)	1.99 (0.24)	3.89
T	4.64 (0.29)	0.668 (0.06)	2.74 (0.27)	4.11
W	4.30 (0.30)	0.621 (0.09)	2.68 (0.18)	4.32
T+W	6.10 (0.58)	0.799 (0.07)	3.63 (0.31)	4.55

FA- Fatty acids ; PUFA- Polyunsaturated Fatty acids

C –intact leaves from control plants (Kept at 25ºC), T-intact leaves from plants exposed to chilling temperature (5ºC) for 24 h; W- wounded leaves from plants kept at the same temperature as control plants, and T+W- wounded leaves from plants exposed to chilling temperatures for 24 h. Values on brackets correspond to the standard deviation values from nine replicates.

Figure 1



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P.oleracea    MASWVLSECGLRPLPKVYNLPRS---KFSSIPSRIRGSPVSPDLKTPYPLKISCCSLSNS 57
A.thaliana    MANLVLSECGIRPLPRIYTTPRS---NFLSNNNKFRPSLSSSSYKTSSSPLSFGLNSRDG 57
D.sophia      MANLVLSECGIRALPRIYTTPRS---SFVSNTTTFSFRPSSSSSSSNTKSLAFRLNSRNG 57
B.juncea      MANLVLSECGIRPLPRIYTTPRS---TFLSN-TTFKLKPSP----------AFRLN---G 43
C.alpina      MATWVLSECGLRPLPRLYSNPRT---RISSSSFPNSVRLAPS--------KIFRPNLSVS 49
G.soja        MATWYHQKCGLKPLAPVIPRPRTG--AALSSTSRVEFLDTNKVVAG-----PKFQPLRCN 53
H.annuus      MAGLVLSGCAIKPFSQSLPIPTK---RFITNPSNINLLHPKD-----------PIFSPNF 46
Z.maize       MARLVLSECSGLAPVRRLRAGRG----AIAARSPPALSAAPR------------RRPASA 44

S.indicum     -REKNWALRVSAPLRVLQVEEEEENKEG-ERVINGGEE-----FDPGAPPPFKLSDIREA 108
P.oleracea    -RERKWEVNVAAPLQVAPPSTAAED-SG-NAVVNGSPEG----FDPAAPPPFKLADIRAA 110
A.thaliana    -FTRNWALNVSTPLTTPIFEESP------LEEDNK--QR----FDPGAPPPFNLADIRAA 104
D.sophia      FFAKNWGLNVSTPLTTPIVEESP------LEEEHEDTQR----FDPGARPPFNLADIRAA 107
B.juncea      FSTKNWALNVTTPLT---VDSSP------PTSEEHETQR----FDPGAPPPFNLADIRAA 90
C.alpina      -RDRRWALKVSAPVGVQFIEEDEQK----VNGINAGESE----FNPGAPPPFTLADIRAA 100
G.soja        LRERNWGLKVSAPLRVASIEEEQKSVDL-TNGTNGVEHEKLPEFDPGAPPPFNLADIRAA 112
H.annuus      HGFSRWAVKVSAPLRIPSIDQQDLDLDLERERISSLDVQEEEIFDAGAPPPFKLADIRAA 106
Z.maize       SIHRDWALRVSAPTRLASAAVEEDKPSAAPGQEEGAAAGG--EFDPGAPPPFGLAEIRAA 102

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P.oleracea    IPKHCWVKDPWRSMSYVVRDVAIVFGLAAAAAYLNNWVVWPLYWFAQGTMFWAPFVLGHD 170
A.thaliana    IPKHCWVKNPWKSLSYVVRDVAIVFALAAGAAYLNNWIVWPLYWLAQGTMFWALFVLGHD 164
D.sophia      IPKHCWVKNPWKSMSYVVRDVTIVFALAAGAAYLNNWLVWPLYWLAQGTMFWALFVLGHD 167
B.juncea      IPKHCWVKNPWKSMSYVVRDVAIVFALAAGAAYLNSWLVWPLYWLAQGTMFWALFVLGHD 150
C.alpina      IPKHCWVKNPWKSMSYVVRDVVVVFGLAAVAAYFNNMVVWPLYWAAQGTMFWALFVLGHD 160
G.soja        IPKHCWVKDPWRSMSYVVRDVIAVFGLAAAAAYLNNWLVWPLYWAAQGTMFWALFVLGHD 172
H.annuus      IPKRCWVKDPWRSMSYVVRDVAIVLGLAAAAAHLNNWLVWPLYWAAQGTMFWALFVLGHD 166
Z.maize       IPKHCWVKDPWRSMSYVLRDVAVVLGLAAAAARLDSWLVWPLYWAAQGTMFWALFVLGHD 162

S.indicum     CGHGSFSNDPKLNSVVGHILHSSILVPYHGWRISHRTHHQNHGHVENDESWHPLSEKIYK 228
P.oleracea    CGHGSFSNDPKLNSVVGHLLHSSILVPYHGWRISHRTHHQNHGHVENDESWHPLSESIYN 230
A.thaliana    CGHGSFSNDPKLNSVVGHLLHSSILVPYHGWRISHRTHHQNHGHVENDESWHPMSEKIYN 224
D.sophia      CGHGSFSNDPRLNSVVGHLLHSSILVPYHGWRISHRTHHQNHGHVENDESWHPMSEKIYR 227
B.juncea      CGHGSFSNDPRLNSVVGHLLHSSILVPYHGWRISHRTHHQNHGHVENDESWHPMSEKIFK 210
C.alpina      CGHGSFSNNAKLNSVVGHLLHSSILVPYHGWRISHRTHHQNHGHVENDESWHPLSEKLYR 220
G.soja        CGHGSFSNNSKLNSVVGHLLHSSILVPYHGWRISHRTHHQHHGHAENDESWHPLPEKLFR 232
H.annuus      CGHGSFSNNAKLNSVVGHLLHSSILVPYHGWRISHRTHHQNHGHVENDESWHPLTEKTFK 226
Z.maize       CGHGSFSNNPKLNSVVGHILHSSILVPYHGWRISHRTHHQNHGHVEKDESWHPLPERLYK 222

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P.oleracea    SLDNMTKKLRFTLPFPLLAYPIYLWKRSPGKSGSHYHPDSDLFNPNERKDIITSTACWSA 290
A.thaliana    TLDKPTRFFRFTLPLVMLAYPFYLWARSPGKKGSHYHPDSDLFLPKERKDVLTSTACWTA 284
D.sophia      GLDKPTRFFRFTLPLVMLAYPFYLWARSPGKKGSHYHPDSDLFLPKEKKDVLTSTACWTA 287
B.juncea      TLDRPTRFFRFTLPLVMLAYPFYLWARSPGKKGSHYNPDSDLFLPKERNDVLTSTACWTA 270
C.alpina      SLDTVTRLTRITLPFPLLAYPFYLWLRSPGKKGSHYHPDSDLFLPNEKKDVITSTVCWTA 280
G.soja        SLDTVTRMLRFTAPFPLLAFPVYLFSRSPGKTGSHFDPSSDLFVPNERKDVITSTACWAA 292
H.annuus      SLDWITRTLRFTLPFPMLAYPFYLWNRSPGKSGSHFDPSSDLFVPAEQKDVITSTICWTT 286
Z.maize       SLDFMTRKLRFTMPFPLLAFPLYLFARSPGKSGSHFNPGSDLFQPTEKNDIITSTASWLA 282

S.indicum     MLALLVGLSFVIGPVQLLKLYGIPYLGNVMWLDLVTYLHHHGHEDKLPWYRGKEWSYLRG 348
P.oleracea    MVALLVGLGFVMGPVQLLELYGVPYVMFVTWLDLVTYLHHHGHDDKLPWYRGEEWSYLRG 350
A.thaliana    MAALLVCLNFTIGPIQMLKLYGIPYWINVMWLDFVTYLHHHGHEDKLPWYRGKEWSYLRG 344
D.sophia      MAALLVCLNFTIGPIQMLKLYGIPYWINVMWLDFVTYLHHHGHEDKLPWYRGKEWSYLRG 347
B.juncea      MAALLVCLNFVMGPMQMLKLYGIPYWINVMWLDFVTYLHHHGHEDKLPWYRGKEWSYLRG 330
C.alpina      MAALLVGLSFVMGPLQVLKLYGIPYVGFVMWLDLVTYLHHHGHEDKLPWYRGKEWSYLRG 340
G.soja        MLGLLVGLGFVMGPIQLLKLYGVPYVIFVMWLDLVTYLHHHGHEDKLPWYRGKEWSYLRG 352
H.annuus      MLALLFGLNFVVGPVQMLKLYGIPYLINVMWLDFVTYLHHHGHEDKLPWYRGKEWSYLRG 346
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P.oleracea    GLTTIDRDYGWINNIHHDIGTHVIHHLFPQIPHYHLIEATEAAKPVLGKYYREPKK-SGP 409
A.thaliana    GLTTLDRDYGLINNIHHDIGTHVIHHLFPQIPHYHLVEATEAAKPVLGKYYREPDK-SGP 403
D.sophia      GLTTLDRDYGLINNIHHDIGTHVIHHLFPQIPHYHLVEATEAAKPVLGKYYREPDK-SGP 406
B.juncea      GLTTLDRDYGVINNIHHDIGTHVIHHLFPQIPHYHLVEATEAAKPVLGKYYREPDK-SGP 389
C.alpina      GLTTLDRDYGWINNIHHDIGTHVIHHLFPQIPHYNLIEATEAAKPVLGKYYREPKK-SWP 399
G.soja        GLTTLDRDYGWINNIHHDIGTHVIHHLFPQIPHYHLVEATEAAKPVFGKYYREPKKSAAP 412
H.annuus      GLTTIDRDYGWINNIHHDIGTHVIHHLFPQIPHYHLIEATEAAKPVLGKYYREPKK-SSP 405
Z.maize       GLTTLDRDYGWINNIHHDIGTHVIHHLFPQIPHYHLIEATEAAKPVLGKYYKEPKN-SGA 401

S.indicum     LPFHLLGDLTRSLKRDHYVSDVGDVVYYQTDPQLTGAEKS--- 447
P.oleracea    LPVYLLGVLWKSLRKDHYVSDTGDVVYYQTDPELLE------- 445
A.thaliana    LPLHLLEILAKSIKEDHYVSDEGEVVYYKADPNLYGEVKVRAD 446
D.sophia      LPLHLLEILAKSIKEDHYVSDEGDVVYYKADPNLYGEVKVRAD 449
B.juncea      LPLHLLGILAKSMKEDHYVSDEGDVVYYKADPNLYGEVKV--- 429
C.alpina      LPFHLLGLVASSMKKDHYVSDEGDVVYYQTDPELSGASK---- 438
G.soja        LPFHLIGEIIRSFKTDHFVSDTGDVVYYQTDSKINGSSKLE-- 453
H.annuus      IPFHLLGELVRSLKKDHYVSDTGDVLYYQTDDKLSKEK----- 443
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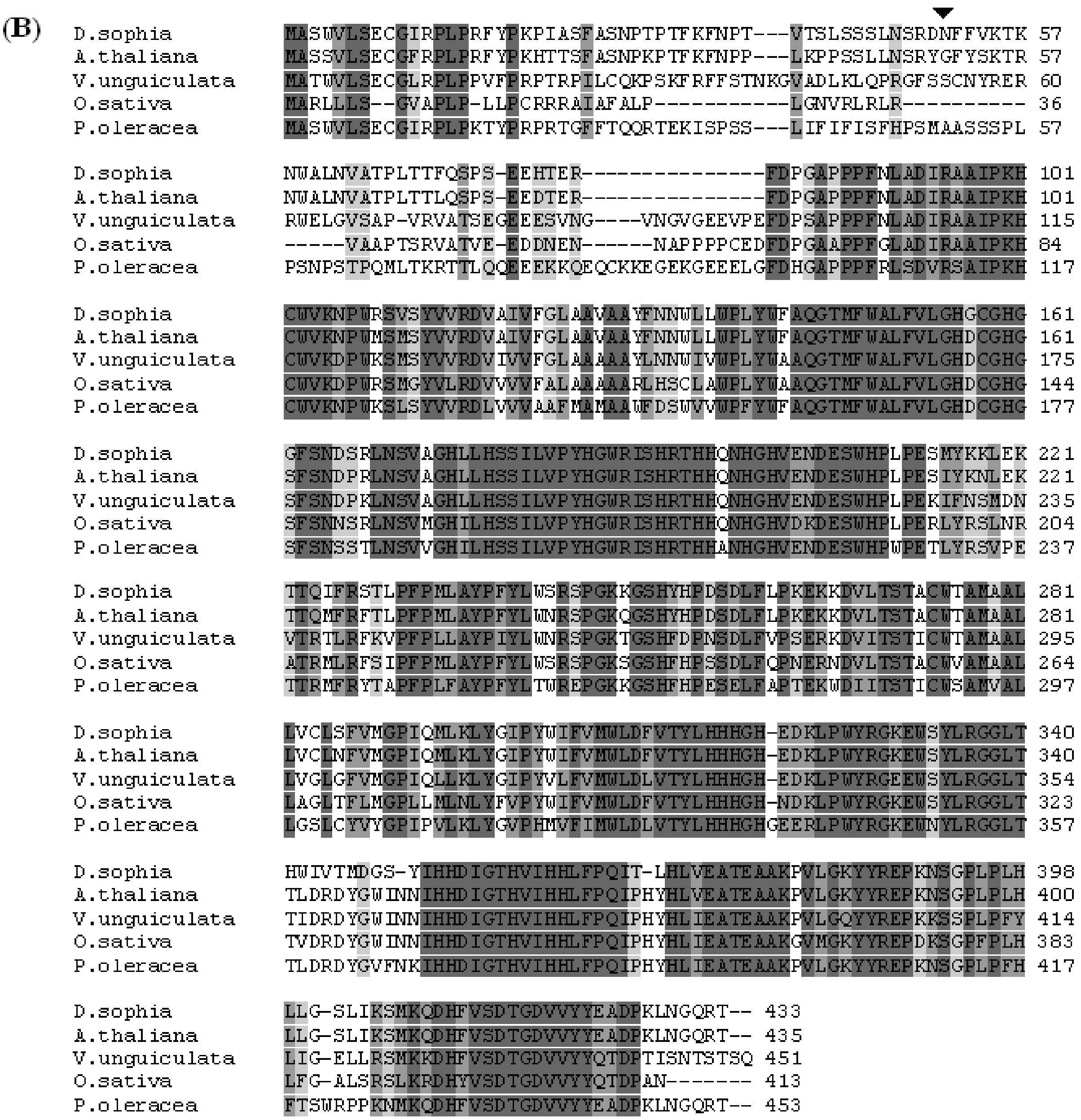

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V.unguiculata   MATWVLSECGLRPLPPVFPRPTRPILCQKPSKFRFFSTNKGVADLKLQPRGFSSCNYRER 60
O.sativa        MARLLLS--GVAPLP-LLPCRRRAIAFALP-----------LGNVRLRLR---------- 36
P.oleracea      MASWVLSECGIRPLPKTYPRPRTGFFTQQRTEKISPSS---LIFIFISFHPSMAASSSPL 57

D.sophia        NWALNVATPLTTFQSPS-EEHTER---------------FDPGAPPPFNLADIRAAIPKH 101
A.thaliana      NWALNVATPLTTLQSPS-EEDTER---------------FDPGAPPPFNLADIRAAIPKH 101
V.unguiculata   RWELGVSAP-VRVATSEGEEESVNG----VNGVGEEVPEFDPSAPPPFNLADIRAAIPKH 115
O.sativa        -----VAAPTSRVATVE-EDDNEN------NAPPPPCEDFDPGAAPPFGLADIRAAIPKH 84
P.oleracea      PSNPSTPQMLTKRTTLQQEEEKKQEQCKKEGEKGEEELGFDHGAPPPFRLSDVRSAIPKH 117

D.sophia        CWVKNPWRSVSYVVRDVAIVFGLAAVAAYFNNWLLWPLYWFAQGTMFWALFVLGHGCGHG 161
A.thaliana      CWVKNPWMSMSYVVRDVAIVFGLAAVAAYFNNWLLWPLYWFAQGTMFWALFVLGHDCGHG 161
V.unguiculata   CWVKDPWKSMSYVVRDVIVVFGLAAAAAYLNNWIVWPLYWAAQGTMFWALFVLGHDCGHG 175
O.sativa        CWVKDPWRSMGYVLRDVVVVFALAAAAARLHSCLAWPLYWAAQGTMFWALFVLGHDCGHG 144
P.oleracea      CWVKNPWKSLSYVVRDLVVVAAFMAMAAWFDSWVVWPFYWFAQGTMFWALFVLGHDCGHG 177

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A.thaliana      SFSNDPRLNSVAGHLLHSSILVPYHGWRISHRTHHQNHGHVENDESWHPLPESIYKNLEK 221
V.unguiculata   SFSNDPKLNSVAGHLLHSSILVPYHGWRISHRTHHQNHGHVENDESWHPLPEKIFNSMDN 235
O.sativa        SFSNNSRLNSVMGHILHSSILVPYHGWRISHRTHHQNHGHVDKDESWHPLPERLYRSLNR 204
P.oleracea      SFSNSSTLNSVVGHILHSSILVPYHGWRISHRTHHANHGHVENDESWHPWPETLYRSVPE 237

D.sophia        TTQIFRSTLPFPMLAYPFYLWSRSPGKKGSHYHPDSDLFLPKEKKDVLTSTACWTAMAAL 281
A.thaliana      TTQMFRFTLPFPMLAYPFYLWNRSPGKQGSHYHPDSDLFLPKEKKDVLTSTACWTAMAAL 281
V.unguiculata   VTRTLRFKVPFPLLAYPIYLWNRSPGKTGSHFDPNSDLFVPSERKDVITSTICWTAMAAL 295
O.sativa        ATRMLRFSIPFPMLAYPFYLWSRSPGKSGSHFHPSSDLFQPNERNDVLTSTACWVAMAAL 264
P.oleracea      TTRMFRYTAPFPLFAYPFYLTWREPGKKGSHFHPESELFAPTEKWDIITSTICWSAMVAL 297

D.sophia        LVCLSFVMGPIQMLKLYGIPYWIFVMWLDFVTYLHHHGH-EDKLPWYRGKEWSYLRGGLT 340
A.thaliana      LVCLNFVMGPIQMLKLYGIPYWIFVMWLDFVTYLHHHGH-EDKLPWYRGKEWSYLRGGLT 340
V.unguiculata   LVGLGFVMGPIQLLKLYGIPYVLFVMWLDLVTYLHHHGH-EDKLPWYRGEEWSYLRGGLT 354
O.sativa        LAGLTFLMGPLLMLNLYFVPYWIFVMWLDFVTYLHHHGH-NDKLPWYRGKEWSYLRGGLT 323
P.oleracea      LGSLCYVYGPIPVLKLYGVPHMVFIMWLDLVTYLHHHGHGEERLPWYRGKEWNYLRGGLT 357

D.sophia        HWIVTMDGS-YIHHDIGTHVIHHLFPQIT-LHLVEATEAAKPVLGKYYREPKNSGPLPLH 398
A.thaliana      TLDRDYGWINNIHHDIGTHVIHHLFPQIPHYHLVEATEAAKPVLGKYYREPKNSGPLPLH 400
V.unguiculata   TIDRDYGWINNIHHDIGTHVIHHLFPQIPHYHLIEATEAAKPVLGQYYREPKKSSPLPFY 414
O.sativa        TVDRDYGWINNIHHDIGTHVIHHLFPQIPHYHLIEATEAAKGVMGKYYREPDKSGPFPLH 383
P.oleracea      TLDRDYGVFNKIHHDIGTHVIHHLFPQIPHYHLIEATEAAKPVLGKYYREPKNSGPLPFH 417

D.sophia        LLG-SLIKSMKQDHFVSDTGDVVYYEADPKLNGQRT-- 433
A.thaliana      LLG-SLIKSMKQDHFVSDTGDVVYYEADPKLNGQRT-- 435
V.unguiculata   LIG-ELLRSMKKDHFVSDTGDVVYYQTDPTISNTSTSQ 451
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Figure 2

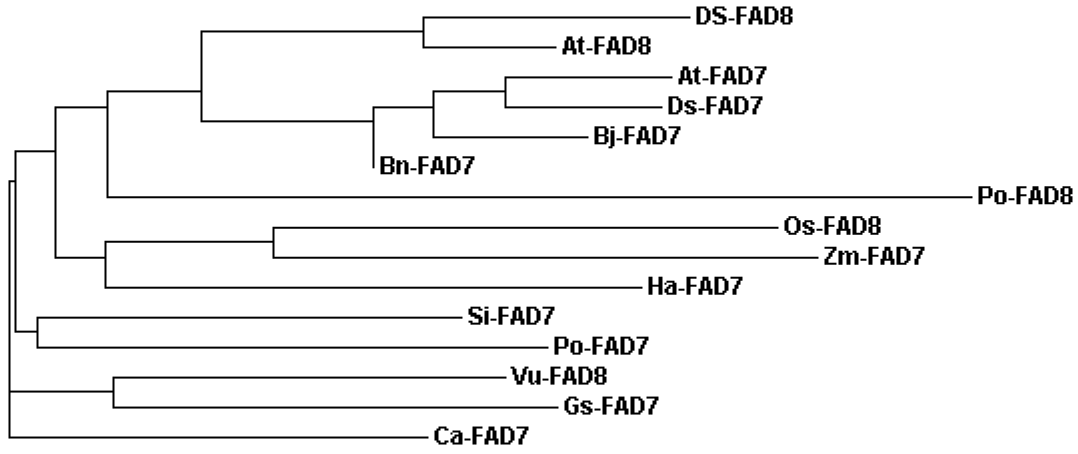


Figure 3

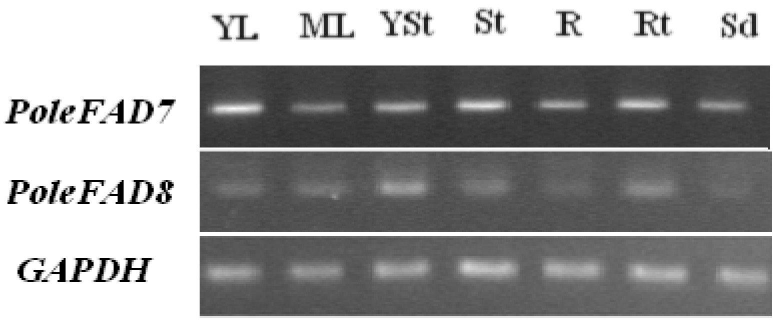
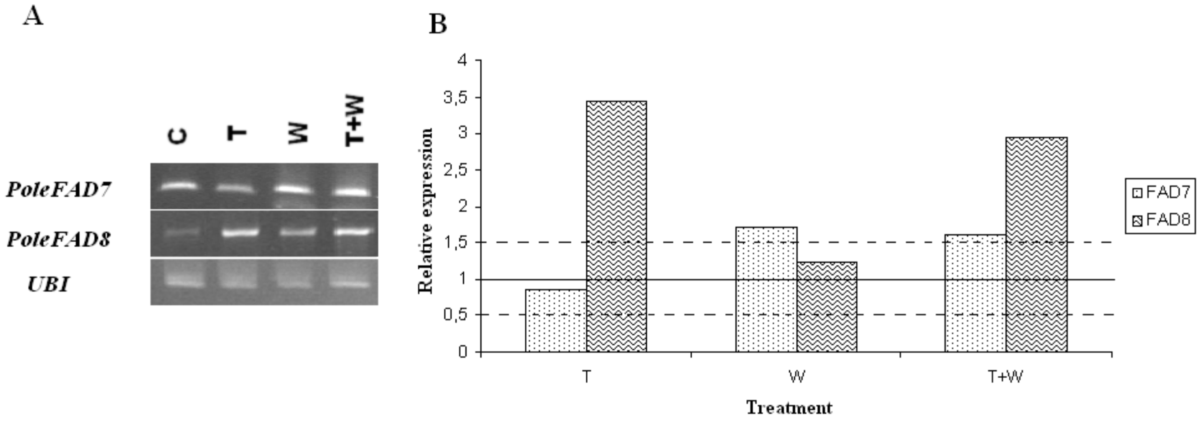


Figure 4


A
C
T
W
T+W
PoleFAD7
PoleFAD8
UBI
B
4
3,5
3
2,5
2
1,5
1
0,5
0
Relative expression
T
W
T+W
Treatment
FAD7
FAD8

PAPER IV

Differential expression of omega-3 and omega-6 desaturase genes and linolenic/linoleic accumulation levels in leaves of *Portulaca oleracea* L., exposed to chloride-salinity treatments.

(Submitted: article ID: Planta-2009-03-0151)

Differential expression of omega-3 and omega-6 desaturase genes and linolenic/linoleic accumulation levels in leaves of Portulaca oleracea L., exposed to chloride-salinity treatments

Journal:	*Planta*
Manuscript ID:	draft
Manuscript Type:	Original Article
Keywords:	omega-3 fatty acids , desaturase genes, salinity stress, Real time PCR, GC-MS

Differential expression of omega-3 and omega-6 desaturase genes and linolenic/linoleic accumulation levels in leaves of *Portulaca oleracea* L., exposed to chloride-salinity treatments

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Abstract

Purslane (*Portulaca oleracea L.*) is a salt-tolerant plant widely used as a vegetable in Mediterranean and Central American countries and in the Philippines, which has been reported to be the a rich source of PUFAs, with a high ω3/ω6 ratio. In this work, we measured the effect of chloride-salinity in plant growth and yield, desaturase genes expression level and linoleic and α-linolenic acids accumulation in purslane leaves. Plants were exposed to 4 levels of chloride-salinity on the root-zone, i.e. 60, 90, 120 and 240 mM NaCl, and relationships between salinity, gene expression and α-linolenic/linoleic levels were followed. No chloride-salinity toxicity signs or plant death were observed in any of the treatments. Yield reduction of 10-15% was observed for 60 mM NaCl; more than 50 % reduction was observed only for 120 and 240 mM NaCl. RT-qPCR analysis showed no significant changes in ω-6 desaturase genes, FAD2 and FAD6 relative expression. However an increase of expression of ω-3 desaturase genes, FAD3, FAD7 and FAD8, at 60 mM and 90 mM NaCl was detected at different phases. Down-regulation of these genes was observed at 120 and 240 mM NaCl levels. The accumulation profiles of LA and ALA measured by GC-MS was correlated with the observed changes in ω-fatty acid desaturase expression. Increase of the expression of ω3 desaturase genes, as a response to moderate salinity conditions, may play a role in purslane ability to subsist in harsh environments.

Key words: omega-3 fatty acids, salinity stress, Real time PCR, GC-MS

Abreviations: ALA- α-linolenic acid; LA – linoleic acid; TFA – Total fatty acids, PUFA – Polyunsaturated fatty acids; NaCl – sodium chloride; FAD – Fatty acid desaturase

Introduction

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stresses are serious threats to agriculture and result in the deterioration of the environment. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Bray *et al*. 2000). Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50 % of arable lands by the year 2050. Abiotic stress led to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al*. 2001, Wang *et al*. 2003). High salinity causes hyperosmotic stress and ion disequilibrium that produces secondary effects or pathologies (Zhu 2001). Fundamentally, plants cope by either avoiding or tolerating salt stress. That is, plants are either dormant during the salt episode or there must be cellular adjust to tolerate the saline environment. The tolerance of salt stressed plants is essentially dependent on their ability to accumulate the inorganic ions within plant tissues in the fastest way to maintain the soil-plant osmotic gradient. This process starts immediately after the occurrence of stress to maintain the water status within the cell (Kamel and El-Tayeb 2004). The cytotoxic ions in saline environments, typically Na^+ and Cl^-, are compartmentalized into the vacuole to minimize cytotoxicity, and used as osmotic solutes (Yokoi *et al*. 2002). Since plant cell growth occurs primarily because of vacuole volume, compartmentalization of these ions facilitates osmotic adjustment that is essential for cellular development. In some species, such as broccoli, cauliflower (De Pascale *et al*. 2005), purslane (Shannon and Grieve 1999), sugar cane (Akthar *et al*. 2001), chickpea (Mor and Manchanda 1992) among others, growth in a chloride salinity system leads to nutrient imbalances or chloride toxicity with subsequent loss of yield.

The extensive genetic diversity for salt tolerance that exists in planta taxa is distributed over numerous genera. Most crops are salt sensitive or hypersensitive plants (glycophytes) in contrast to halophytes, which are native flora of saline environments. Purslane is classified by the Mass-Hoffman (1977) model as a moderate-tolerante plant to salinity, and it is an important vegetable in southern Europe, Mediterranean countries and Asia. Purslane has long been a food source for humans and fodder for livestock, although the interest in cultivating purslane as a food crop has only increased since its identification as a rich source of ω3 PUFAs (Kumamoto *et al*. 1990; Omara-Alwala *et al*. 1991; Simopoulus 2004) and anti-oxidants such as α.tocopherol, ascorbic acid, β-carotene and gluthathione (Simopoulos 2004; Simopoulos *et al*. 1992). Alternative sources of PUFA are desirable, and the concept of obtaining them from higher plants in commercial and sustainable quantities is particularly attractive.

PUFAs can be defined as fatty acids of 18 carbons or more in length, containing two or more double bonds. These double bonds are inserted by specific fatty acid desaturase enzymes that have been the subject of intense research in recent years (Shanklin and Cahoon 1998; Napier *et al*. 1999). Linoleic and α-linolenic acids are essential fatty acids synthesized in plant tissues from oleic acid by the introduction of double bonds between the existing double bond and the terminal methyl group by the sequential action of Δ12 and Δ15 desaturases. Two genes have been identified that code for Δ12-fatty acid desaturase, FAD2 and FAD6, the enzyme that insert Δ12 double bound into oleic acid precursors to produce linoleic acid. Three distinct desaturases have been characterized that can insert the Δ15 double bond into linoleic acid precursors to produce linolenic acid, i.e. two ω-3 desaturase plastid enzyme (*FAD7 and FAD8*), which uses the terminal methyl group as a reference point, and secondly an extra-plastid oleate Δ15 desaturase (*FAD3*) (Ohlrogge and Browse 1995).

It has been described that environmental conditions modulate the ALA/LA (C18:3/C18:2) ratios of membrane and storage lipids through effects on desaturase enzyme activity and possibly gene expression (Hamada *et al*. 1996; Zhang *et al*. 2005; Im *et al.* 2002). The proven ability of purslane to accumulate higher amounts of ALA than LA acids in leaves and its tolerance to saline environment makes it a valuable crop to consider, providing a desirable vegetable source for PUFAs in the diet. The identification of genes that control and facilitate the accumulation of these fatty acids in purslane leaves, and studying the way that enviornmental conditions affect these genes's expression, would provide useful tools for increasing the concentration of LA and ALA in plant tissues by means of either improved selection or genetic modification. This work aims at an examination of the response of purslane in terms of plant yield, PUFA content, and ω-3 and ω-6 fatty acid desaturase gene expression to imposed chloride-salinity treatments..

Material and Methods

Plant materials and growth conditions

Twenty-one-day old purslane seedlings were transplanted into a closed hydroponic system under greenhouse conditions. Nitrogen at 200 µg ml^{-1} was provided as NO_3^- and NH_4^+forms to yield a NO_3^-:NH_4^- ratio of 1:1. The nutritive solutions also contained macronutrients (in mM) 31 P, 207 K, 200 Ca, 48 Mg, and 64 S, and the micronutrients (in µM) 2 Na, 50 Cl, 24 B, 2 Mn, 2 Zn, 0.5 Cu, 0.5 Mo, and 50 Fe-EDDHA. Four saline treatments were applied: 60, 90, 120, and 240 mM; the control corresponded to the base nutritive solution. Eight plants were randomly placed in each tank, and two tanks were used for each treatment, making a total of 16 plants for each treatment. The nutrient solution in the hydroponic system was aerated for 15 minutes every two hours using a time-controlled air bubbler. The solution pH was monitored at 2-day intervals and maintained at 6.6-6.8 by adding 0.5 M HCl or 0.5 M NaOH, as needed. The conductivity of nutritive solutions was also monitored to verify the concentration of soluble ions. Half of the nutritive solution was replaced every week, to ensure the nutrient level in the nutritive solutions. Two plants (one from each tank) were randomly selected and harvested after 7, 15, 21, 28, and 42 days of treatment, hereby designated by stages 2 to 6. Stage 1 corresponded to the seedling stage (21days-old), when the plants were transferred to the hydroponic tanks. The plants were collected and analyzed at this stage. Only adult mature leaves were collected which corresponded to the third, fourth and fifth pairs of leaves, counting from the base of the shoot (aerial parts). The variation in physiological parameter, such as plant height, leaf blade length, leaf area, and plant fresh weight were determined during the treatments, at all harvest times. Leaf area was determined based on the leaf length (l) and width (w), considering the geometric shape of an elipse ($A= \pi lw$). The relative yield of the crop was determined on the basis of shoot fresh weight, using the following equation: $Y_t=Y/Y_m$, where Y represents the absolute yield; Y_t represents the relative yield of a test crop rooted in a series of incrementally saline environments, and Y_m designates the yield of the crop when grown in a root zone free of salinity (Mass and Hoffman 1977; Mass 1990). The variation in physiological parameter during the treatments was analysed using the SAS (Statistical Analysis System) software. ANOVA analysis was used for the analysis of data, and significant differences between means were determined by least significant difference (LSD), with a significance level of 0.05. The samples were analysed in triplicate.

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from 100 mg of frozen powdered leaves using the PureScript Cell & Tissue Trial Kit (Gentra Systems, USA), according to manufacturer's instructions. Two different samples were used and were treated with RNase-free DNaseI (Invitrogen, USA), at 37ºC for 20 min, and purified using the phenol-chloroform extraction procedure. To verify RNA integrity, 500 ng of total RNA of each sample was electrophoresed on a 1% agarose gel stained with ethidium bromide, and total RNA was quantified spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (Saveen Werner, Sweden). For semi-quantitative gene expression analysis, a first strand cDNA was synthesised from 1 µg of total RNA by the oligo-dT priming method using SuperScriptIII™ reverse transcriptase (Invitrogen, USA), following the manufacturer's protocol.

RT-qPCR protocol

The differential expression of target genes can be analysed using semi-quantitative real time PCR (RT-qPCR. Expression of the target gene was normalized relative to that of a reference gene, ubiquitin (UBI) which express stable levels of transcripts in the tissues being compared, under the assay conditions. Specific primers for target genes were designed, based on the nucleotide sequence of each gene, to amplify a partial central region of PoleFAD2 (EU872254), PoleFAD3 (Bankit1189465), PoleFAD6 (EU376530), PoleFAD7 (ABL61262) and PoleFAD8 (EU872255) genes isolated from purslane (*Portulaca oleracea*) leaves (Table 1) and were used in a final concentration of 2 µM in the PCR mixture. The equivalent of 40 ng of reverse transcription products (2 µl) was used as the template in a 20 µl reaction mixture containing 2 µM primer mix (Table 1), and 10 µl 2x SYBR green master mix (Applied BioSystems).

Table 1

The RT-qPCR of reference and target gene transcripts in cDNA samples were conducted in an ABI model 7500 thermocycler (Applied Biosystems). All samples were amplified in duplicate assays under the following conditions: 95°C for 10 min for 1 cycle, followed by 40 cycles of

95°C for 15 s and 1 min at 60 °C. The PCR products for each primer set were also subjected to melt-curve analysis. The melt-curve analysis was done from 60 to 95°C. RT-qPCR results were analysed with the sequence detection software SDS version 1.1 (Applied Biosystems). Direct detection of the PCR product was measured by monitoring the increase in fluorescence caused by the binding of SYBR green dye to double-strand DNA. A standard fluorescence threshold was set to a ΔRn of 0.2 on the log fluorescence scale to determine the fractional cycle number (Ct value). For each cDNA sample, relative expression levels of each protein coding gene were normalized by reference to the ubiquitin gene assay. The transcript abundance ratio of the target gene to reference gene was determined by the following equation: relative expression = $(E_{ref})^{\Delta Ct\ ref} / (E_{target})^{\Delta Ct\ target}$ (Pfaffl, 2001; Czechowski, Bri, Stitt, Scheible and Udvardi, 2004), where E_{ref} and E_{target} are the efficiencies of the primers for the reference and the target gene, respectively, and ΔCt_{ref} and ΔCt_{target} are the difference between the mean Ct value of reference and target genes for the control plants and the plants exposed to saline treatments, respectively. The RT-qPCR protocol was optimized by determining the optimal primer concentration and primer efficiency. Primer amplification efficiency was determined with data from amplification plots of individual reaction tubes generated during RT-qPCR. Fluorescence data generated from each reaction were used to determine the slope of the exponential phase of the amplification curve of cycle number versus log fluorescence. We consider an up-regulation of the target genes for relative expression values above 1.5 and a down-regulation for relative expression values below 0.5. The RT-qPCR products were separated by 1% agarose gel electrophoresis. The RT-qPCR fragments were cloned in *E. coli* and sequenced by Eurofins MWG GmbH (Germany), confirming the identity of the amplified products.

Analysis of fatty acid content

Fatty acid analyses were done on identical tissue samples as those used for RNA extraction. The one-step extraction-methylation procedure applied was based on the protocol by Vrinten *et al.* (2005). 0.5 g of frozen leaf tissue was suspended in 2 ml of 3 N methanolic HCl and incubated at 85ºC for 90 minutes. Methylated fatty acids esters (FAMEs) were extracted with 2 x 2 ml of hexane. The hexane layer was transferred to a new tube and evaporated to dryness in a current of helium gas. The extract was resuspended in 1 ml of hexane for fatty acids analysis. The extracts were diluted 10 times into hexane and immediately analysed by GC-MS. The FAMEs were separated by gas chromatography using an Agilent 6890 Series equipped with a column SGE 25QC2/BPX70 (25 m × 220 μm × 0.30 μm) and connected to a mass detector Agilent 5973 Network. The samples were injected with an automatic injector Agilent 7683 Series in the split less mode at 250 ºC. The oven was programmed to hold at 130 ºC for 1 min, increase to 180 ºC at 25 ºC/min, and finally hold for 5 min and increase to 230 ºC at 2.5 ºC/min. Helium was used as the gas carrier with a velocity of 59 cm/s. The temperature of the ionization source and the quadruple were 230 ºC and 150 ºC, respectively. Fatty acid methyl esters were identified by comparing the GC retention times with those of a mixture of standard fatty acids methyl esters (FAMEs) Mix C14-C22 (Supelco). Heneicosaenoic acid (C21:0) was used as the internal standard for quantification.

Results /Discussion

Physiological parameters

Purslane has a relatively short life cycle. The seeds germinate in large number on the soil surface after irrigation or rain, when soil temperature reaches 15ºC (60ºF); seedlings grow fast and reach the adult state in 4 to 6 weeks, after which flowering occurs and seed production initiates shortly thereafter. The mature seeds are released 14 to 16 days after flowering (Rashed *et al.* 2003, Matthews *et al.* 1993, Kedshen and will 1987). In this work we exposed 21-day-old seedlings of purslane to environmental salinity, in the range of 60 to 240 mM NaCl, for a 42 day treatment period. The shoot length and plant fresh weight were determined for each harvest, as well as, leaf central blade length and leaf area.

Purslane is known to be moderately tolerant to salinity with a threshold value, in terms of saturated soil (ECe) of 6.3 dS m^{-1} (Grieve 2000, Kumamoto *et al.* 1990, Mass and Hoffman 1977). This value roughly corresponds to a NaCl concentration of 60 mM in the root-zone, so it was no surprise that plants exposed to 60 mM NaCl treatment showed similar development to control plants during the assay, with a minimal variation in the measured physiological parameters (Figs. 2A-D).

Figure 1 / Figure 2

The plant exposure to saline treatments led to an evident decrease on plant development with a reduction on plant size and secondary shoot development (Fig. 1). At salinity levels superior to 60 mM NaCl, all physiological parameters were reduced or kept constant during treatments.

Although exposed to saline treatments, all purslane plants completed the life cycle with the production of more than one batches of seeds and no plant death or tissue necrosis was registered. It seems that the plant somehow recuperates from the effects of the stress and adapts to the environmental salinity, having a secondary vegetative growth at 28 days of exposure (Figs. 2A-D), right after the maturation and release of the first group of seeds.

All plants flowered and produced seeds in all salinity treatments, although these events occurred a week prior to the control plants in the plants exposed to intermediate and higher levels of salinity, i.e. 90, 120 and 240 mM NaCl. It is possible that this reduction in plant life cycle may be an adaptive response of purslane to environmental salinity. Plants usually activate their flowering program during a transient period of threatening environmental conditions. There are a variety of stress factors able to promote flowering including pathogen infection, drought, extreme temperatures and high levels of radiation (Martinez *et al*. 2004).

Figure 3

Significant reduction on plant yield was only observed for salinity levels superior to 60 mM NaCl, placing this plant in the salt-tolerant group of plants with one of the highest tolerance level together with artichoke (Shanon and Grieve 1999). A relative yield reduction of 40, 70 and 95 % was registered for 90, 120, and 240 mM NaCl treatments, respectively (Fig. 3). These findings are in agreement with the findings of Kumamoto *et al*. (1990), which reported similar yield reduction values in purslane cultures for the same order of NaCl concentrations. A decrease in culture yield was also reported in tomato cultures (*Lycopersicon esculentum* Mill.) (D'Amico *et al*. 2003) and in two forage sorghum genotypes (*Sorghum bicolor* L.) (Lacerda *et al*. 2003) when exposed to saline environments.

These results reinforce the value of this plant as a potential culture to be cultured in areas with soil-salinization problems, whereas other more sensitive cultures can not be produced.

Salinity effect on fatty acid composition in purslane leaves

Purslane is very rich in fatty acids compared to other leafy vegetables, such as spinach, lettuce and mustard, with a total lipid content of 8.5 mg TFA/g fresh weight (Xiang *et al*. 2005, Simopoulos *et al*. 1992). The best growth stage for harvest has been established to be the "14-true leaf stage" (Ezekwe *et al.* 1999), which corresponds to the adult stage of plant development, just before flowering. The high total lipid content of purslane leaves observed in the present study was in agreement with the findings of Mangalan *et al.* (1989), and Ezekwe *et al.*(1999). Teixeira and Carvalho (2008) had previously reported an increase of TFA in purslane leaves exposed to 60 mM NaCl for a relative short period (15 days). In this study we did not observe an increase on TFA in purslane leaves, although the plants treated with 60 mM NaCl had similar TFA content as the control plants. This data suggests that for salinity levels below the plant salinity threshold value (EC_e = 6.3 dS m^{-1}), the plant's ability to synthesize and accumulate lipids in leaf tissues will not be compromised. Higher salinity treatments led to a decrease on TFA content, in particular in the early stages of treatment (Fig. 4), which is in agreement to the findings of Teixeira and Carvalho (2008).

Environmental stresses such as cold, heat, drought and salt induce changes in fatty acid composition, mainly in the content of α-linolenic acid (ALA C18:3). Both salt and drought stresses were found to reduce the amount of ALA, for example in rape leaves, cruciferous herbs (*Crambe* sp.), pea (*Pisum sativum*), wheat (*Triticum aestivum*), soybean (*Glycine max*), the legume *Pachyrhizus ahipa*, and in salt-tolerant but not salt sensitive *citrus* cells (Zhang *et al.* 2005).

Purslane has been reported as an important source of polyunsaturated fatty acids, in particular ALA, present in levels 4-5 times higher than LA (Palaniswany *et al.* 2001, Ezekwe *et al.* 1999, Simopoulus *et al.* 1992). In this work, similar fatty acid proportions were observed in the control plants; the polyunsaturated lipids fraction corresponded to 75 to 80 % of the total fatty acids, the largest contribution being α-linolenic (ALA) acid, which accounted for 54 to 69 % of the total lipid content (Table 2). This result was also in agreement with the findings of Liu *et al.* (2000), who reported that ALA was the major contributor to the polyunsaturated fatty acid fraction in leaves and seeds of Australian purslane varieties. Plants exposed to 60 mM NaCl had the highest levels of LA and ALA acids, ranging from 0.34 to 1.19 mg LN/g fresh leaves, and 1.55 to 5.41 mg ALA/g fresh leaves (Table 2), with a corresponding value of 13-16% and 59-68.5% of TFA, respectively.

Table 2

Salinity treatments did not significantly affected LA content in purslane leaves. Although some variation in absolute amount of LA was observed within the salinity treatments at different stages of plant development, the LA proportion in the fatty acid mixture was conserved, with levels ranging from 12 to 19 % of TFA (Table 2).

On the other hand, ALA synthesis was affected by environmental salinity, illustrated by the reduction on ALA content in the more severe salinity treatments (Table 2). A reduction on absolute amount of ALA was registered for increasing salinity values, but ALA/LA ratios were in kept in the same order of value as control plants in the same stage of development, except for the most severe treatment, whereas the lowest LNA/LA ratios were obtained (Table 2). It is possible that the significant amount of compounds with anti-oxidant action, such as betalains and β-carotenes, present in purslane tissues (Simopoulos *et al*. 1992) may have a protective effect against oxidation, which could explain the relative high ratio of LNA/LA acids, even when high saline treatments are applied.

The ability to adjust membrane lipid fluidity by changing levels of unsaturated fatty acids is a feature of stress acclimating plants provided mainly by the regulated activity of fatty acid desaturases. Free ALA, released from the membranes through lipase activity, is it self a stress signal (Upchurch, 2008). The reduction of ALA accumulation in leaf tissue can be related to the lower expression level of ω-3 desaturase genes observed in more severe treatments (Figs. 5C-E). Also, the release of ALA from plant membrane lipids by leaf senescence and/or stress activated lipases is thought to provide the substrate for lipoxygenase and subsequent octadecanoid (oxilipin) pathway synthesis of jasmonic acid and methyl jasmonate (Padham *et al*. 2007). It is possible that a fraction of produced ALA is being mobilized to produce other stress signal related molecules, which would account for lower levels of ALA in cell membranes. Since a long treatment period (42 days) was applied on this study, we would expect a reduction in PUFA synthesis due to leaf senescence in the final development stages. However, no reduction on PUFAs leaf content in the latest stages of plant development was observed, in fact higher values of total lipids, LA and LNA absolute content were registered at 28 and 42 days of treatment. This data suggest the this plant, after a adaptation period, is able to recuperate from the induced stress and reestablish not only vegetative growth (Fig. 2), but also its metabolic functions, such as lipids biosynthesis.

In general, these results suggest that the exposure of purslane to salinity may slow down the lipid metabolisms, illustrated by the decrease in the absolute values of TFAs and PUFAs (LA and ALA) in the early stages of plant development. However PUFA biosynthesis is not completely inhibit, since ALA/LA ratios are essentially maintain throughout the assay. This ability of maintaining the insaturation levels in the membranes, despite the increasing environmental salinity, may play a role on this plant tolerance to salt stress. It seems that the relative proportion of LA and LNA in cell membranes, rather than the absolute amount, is an important factor for purslane survival under saline conditions.

Transcript levels of target genes across salinity

Relative expression of genes involved in LA and ALA synthesis in purslane plants exposed to different saline treatments was studied by RT-qPCR. We performed RT-qPCR experiments with cDNA obtained from total RNA samples extracted from adult mature leaves of purslane. In this work, the relative expression of two ω6-desaturase genes: FAD2 and FAD6, and three ω3-desaturase genes: FAD3, FAD7 and FAD8, was followed throughout the assay. The relative expression of both, microsomal and plastid desaturase genes was influenced by the treatments and stage of plant development (Fig. 5).

Figure 5

Abiotic stresses can affect fatty acid desaturase genes expression. Evidences of the importance of dienoic fatty acids in the tolerance to salt and drought were shown by the introduction of two sunflower (*Helianthus annus*) ω-6 desaturase genes (FAD2-1, FAD2-3) into yeast, which resulted in the production of dienoic fatty acids, and increased unsaturation index and fluidity, led to higher levels of tolerance to NaCl and to freezing (Rodríguez-Vargas *et al*. 2007). Environmental salinity and stage of plant development had an effect on the relative expression of ω3 and ω6 fatty acid desaturase genes in purslane. A minor variation on the relative expression of FAD2 and FAD6 desaturase genes (Figs. 5A-B) was detected in treatments. Relatively constant levels of leaf LA content was observed in all treatments (Table 2) which is in accordance to the levels of gene expression (Fig. 5A-B) Although an overexpression of purslane FAD2 and FAD6 genes or a significant increase on the LA content were not detected, LA/OA index increased with increasing NaCl levels, which may indicate an adjustment of the membrane fatty acid composition to a higher level of unsaturation. Also it is possible that LA is used as a substrate for the production of ALA, in order to maintain higher unsaturation levels in the membranes.

The expression of each of the ω-3 fatty acid desaturase genes, the chloroplast-localized (FAD7, and FAD8) and the ER-localized (FAD3) forms appears to be regulated in a complex way in response to changes in the enviornment or other stress-inducing factors. In many plant systems, salt/drought stress results in reduced ALA, therefore it would be reasonable to assume that this reduction is a component of their defence mechanism against the stress, rather that one aspect of the damage (Upchurch, 2008). Some examples are rape leaves, cruciferous herbs (*Crambe* sp), pea (*Pisum sativum*), wheat (*Triticum aestivum*), soybean (*Glycine max*), in the legume *Pachyrhizus ahipa*, and in salt-tolerant, but not salt sensitive citrus cells (Matos *et al*. 2002, Surjus and Durand 1996,Dakhma *et al*. 1995; Olsson 1995,Gueta-Daham *et al*. 1994, Mansour *et al*. 1994, Francois and Kleiman 1990).Conversely, there are few reports where ALA increases, particularly in chloroplast lipids, under drought stress (Repellin *et al*. 1997, Sgherri *et al*. 1996), raising the question whatever the increase or the decrease of ALA contributes to stress tolerance. Experiments with transgenic tobacco cells and plants demonstrated that the overexpression of ω-3 desaturases, increases ALA accumulation and tolerance to salt and drought stresses (Zhang *et al*. 2005, Im *et al*. 2002).

In our experiments, we also observed an up-regulation on the expression of ω3-fatty acid desaturase genes in the mild saline treatment, 60 mM NaCl, more evident for the plastid gene FAD8, that showed a 2 to 4-fold increase in expression compared to control plants (Fig. 5E). An up-regulation in FAD7 relative expression by 1.5-fold and 2-fold at 15 and 21 days of treatment was also observed. In general, treatments with NaCl concentrations above 90 mM had a negative effect on the expression levels of all ω3 desaturase genes, with relative expression values inferior to control plants (Figs. 5C- E).

In contrast, to the plastidial genes, the expression of the ER ω-3 desaturase gene (FAD3) is thought to be regulated through the synergistic and antagonistic interaction of plant hormones, such as auxin, cytokinin, and abcisic acid, and the tissue specificity of the expression of this gene is further modified in accordance with the growth phase in plant development (Matsuda *et al.* 2001, Zou *et al.*1995, Yamamoto 1994). Relative expression of the microsomal form, FAD3, was up-regulated by exposure to mild salinity treatment and was kept constant during the majority of the assay (Fig. 5C). A 2-fold and a 1.5-fold increase in the FAD3 expression level was registered for 90 and 120 mM NaCl treatments, respectively, at 21 days of treatment (Fig. 5C). The same relative expression variation pattern was observed in the leaves of plants exposed to the highest level of salinity, but lower levels of expression were observed.

Although a direct correlation between expression levels of the desaturase genes and the accumulation pattern of LA and ALA in purslane leaves is not present, a parallel increase of these fatty acids was observed when the gene expression levels increased, particularly in plants exposed to the mild saline treatment (Table 2).

Our findings suggest saline environments had a direct effect on the transcriptional regulation of ω-3 desaturase genes expression, particulary the plastid forms FAD7 and FAD8, which was later reflected on ALA accumulation in purslane leaves. Saying so, this plant seems to be particularly suitable as a crop for culture in moderated (up to 60 mM NaCl) saline soils.

Conclusions

To conclude, the results of this work show that *Portulaca oleracea* has a high potential for use as a highly nutritional vegetable for both animal (fodder) and human consumption. Salinity significantly affected growth and yield of purslane plants, although a significant yield reduction was only observed in plants exposed to the highest levels of salinity. Linoleic and α-linolenic acids accumulation was also affected with a slight decrease for high levels of salinity, but overall the levels of these fatty acids in purslane leaves were conserved once the plant reached the adult stage. A direct correlation between the ω-6 and ω-3 desaturase genes relative expression level and the linoleic and linolenic acids content in purslane leaves was not observed, although the higher content of these fatty acids was obtained when an increase on gene expression was also observed. FAD2 and FAD6 genes level of expression was not significantly affected by the imposed saline treatments, although a slight increase on expression was observed. On the other hand, relative expression of both, microsomal and plastid forms of ω3-fatty acid desaturase genes, was affected though by different rates and in different phases of plant development. Mild and moderate salinity conditions led to an up-regulation of ω-3 desaturase genes and an increase in α-linolenic accumulation on purslane leaves, in particular in the latest stages of plant development. Extreme salinity conditions led to a reduction on the relative expression levels of ω3-fatty acid desaturase genes, more evident for the plastid forms, FAD7 and FAD8. According to this study, purslane is an interesting PUFA-source-vegetable suited for growing in soil with a mild and moderate degree of salinity, without significant detriment to its yield or nutritional properties.

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Tables

Table 1. Sequence specific primers sets used in RT-qPCR assay and expected size of the amplified DNA fragment

Gene	Primer designation	Primer sequence	Size of amplified fragment
PoleFAD2	FAD2 RT-PCR fw	5`- GGG TCA TTG CCC ACG AAT GCG GCC A - 3’	181 bp
	FAD2 RT-PCR rv	5’- CGG CCT GGT GGG TTG TTT AGG TAC TT -3’	
PoleFAD3	FAD3 RT-PCR fw	5`-CCTACCCTTGACCCAGAACAGGGTT-3`	174 bp
	FAD3 RT-PCR rv	5`-GATGAAAACACAGACAATGCAGC-3`	
PoleFAD6	FAD6 RT-PCR fw	5’- GCC GTG CCA GAA GGG TTT CGG TA-3’	197 bp
	FAD6 RT-PCR rv	5’- CAC CTT AGG AAG GGA ACT GAT -3’	
PoleFAD7	FAD7 RT-PCR fw	5`-GAGGTTCCCCTGTTTCCCCGGAT-3`	175 bp
	FAD7 RT-PCR rv	5`-AATGCGGTGGTCAATGGGTCGCC-3`	
PoleFAD8	FAD8 RT-PCR fw	5`-CTCCCATCTTCTCTCATTTTCATT-3`	193 bp
	FAD8 RT-PCR rv	5`-TTAGGGTTTGATCATGGGGCACC-3`	
UBI[a]	UBI RT-PCR fw	5`-GGCATGCAGATCTTTGTGAAGAC-3`	180 bp
	UBI RT-PCR fw	5`-GGGATACCCTCCTTGTCCTG-3`	

[a] – Housekeeping gene

Table 2. Selected fatty acids accumulation: oleic (OA), linoleic (LA), and linolenic (ALA) content in purslane leaves (mg FAME/ g fresh weight); LA/OA and ALA/LA ratios in purslane plants exposed to chloride-salinity stress conditions over 42 days of treatment. Values in brackets correspond to the proportion, in percentage, of OA, LA, and LNA, of the total fatty acid content.

NaCl (mM)	Treatment (days)	OA (mg/g leaves)	LA (mg/g leaves)	ALA (mg/g leaves)	LA/OA	ALA/LA
0	0	0.20 (3.5 %)	0.34 (12.8 %)	1.55 (57.4 %)	1.69	4.56
	7	0.26 (3.6 %)	1.12 (15.6 %)	4.25 (58.9 %)	4.34	3.79
	15	0.21 (3.0 %)	0.56 (16.2%)	2.34 (68.5 %)	4.18	4.18
	21	0.26 (3.0 %)	1.03 (14.6%)	4.37 (62.7 %)	4.02	4.24
	28	0.36 (4.0 %)	1.30 (15.4 %)	5.01 (59.3 %)	3.65	3.85
	42	0.26 (3.5 %)	0.94 (12.8 %)	4.64 (63.7 %)	3.57	4.94
60	0	0.20 (3.5%)	0.34 (12.8 %)	1.55 (57.4 %)	1.69	4.56
	7	0.23 (3.7 %)	0.94 (15.0 %)	3.71 (59.0 %)	4.14	3.95
	15	0.11 (3.0 %)	0.58 (16.6 %)	2.41 (66.0 %)	5.52	4.16
	21	0.22 (3.0 %)	0.65 (14.7 %)	4.8 (63.1 %)	2.95	7.38
	28	0.34 (4.0 %)	1.25 (15.4 %)	4.82 (59.4 %)	3.70	3.86
	42	0.38 (4.5 %)	1.19 (13.8 %)	5.41 (63.0 %)	3.10	4.55
90	0	0.20 (3.5 %)	0.34 (12.8 %)	1.55 (57.4 %)	1.69	4.56
	7	0.15 (3.3 %)	0.71 (15.6 %)	2.79 (61.4 %)	4.78	3.93
	15	0.10 (2.8 %)	0.70 (17.8 %)	2.69 (58.5 %)	7.11	3.84
	21	0.14 (2.8 %)	0.92 (15.2 %)	3.78 (62.3 %)	6.62	4.11
	28	0.16 (3.0 %)	0.93 (15.3 %)	3.68 (60.5 %)	5.65	3.96
	42	0.36 (4.0 %)	1.16 (14.0 %)	5.37 (63.1 %)	3.26	4.63
120	0	0.20 (3.5 %)	0.34 (12.8 %)	1.55 (57.4 %)	1.69	4.56
	7	0.14 (2.7 %)	0.76 (15.4 %)	3.06 (62.0 %)	5.60	4.03

	15	0.14 (2.8 %)	0.72 (18.0 %)	2.69 (52.5 %)	5.16	3.74
	21	0.13 (3.2 %)	0.76 (15.1 %)	3.11 (62.0 %)	5.78	4.09
	28	0.28 (4.0 %)	1.07 (15.3 %)	3.86 (54.7 %)	3.78	3.61
	42	0.35 (3.5%)	1.13 (15.3 %)	4.76 (63.4 %)	3.27	4.21
240	0	0.20 (3.5 %)	0.34 (12.8 %)	1.55 (57.4 %)	1.69	4.56
	7	0.12 (2.8 %)	0.73 (17.3 %)	2.46 (58.1 %)	6.15	3.37
	15	0.13 (2.4 %)	0.87 (17.4 %)	2.7 (53.9 %)	6.93	3.10
	21	0.25 (3.0 %)	0.84 (18.6 %)	2.51 (56.0 %)	3.41	2.99
	28	0.26 (4.0 %)	1.26 (17.3 %)	4.12 (56.4 %)	4.76	3.27
	42	0.27 (4.0 %)	1.34 (17.7 %)	4.24 (55.7 %)	4.93	3.16

Figures

Figure 1: Purslane plants after 21 days of exposure to chloride-salinity treatments.

Figure 2: Physiological plant growth parameters determined on purslane plants exposed to chloride-salinity treatments during 42 days of treatment. A- Purslane development stages during saline treatment of control plants; B-Shoot length; C- Shoot fresh weight; D- Leaf central blade length; and E- Leaf area.

Figure 3: Relative yield of purslane plants exposed to chloride-salinity treatments during 42 days of treatment. The relative yield was determined on shoot fresh weight basis, considering the fresh weight of control plants the maximum yield value.

Figure 4. Total fatty acid contents (mg TFA/g fresh leaves) of purslane leaves from plants exposed to chloride-salinity during 42 days of treatment.

Figure 5: Relative expression of target genes in leaves of *P. oleracea* plants subjected to different saline stress treatments. Assays of transcript levels for each target gene were normalized by ubiquitin gene expression for ΔCt value calculation. Target gene expression level in plants exposed to the treatments was also normalized by the expression level of each correspondent gene in the leaves of control plants. The continuous line corresponds to a relative expression level of 1, in wich the target gene expression from the plant exposed to the saline treatment equals the target gene expression in the control plants. The interrupted lines correspond to the limits on the

expression level to consider an up-regulation (RE ≥1.5) or a down-regulation (RE≤0.5) of the target genes.

Figure 1

Figure 2

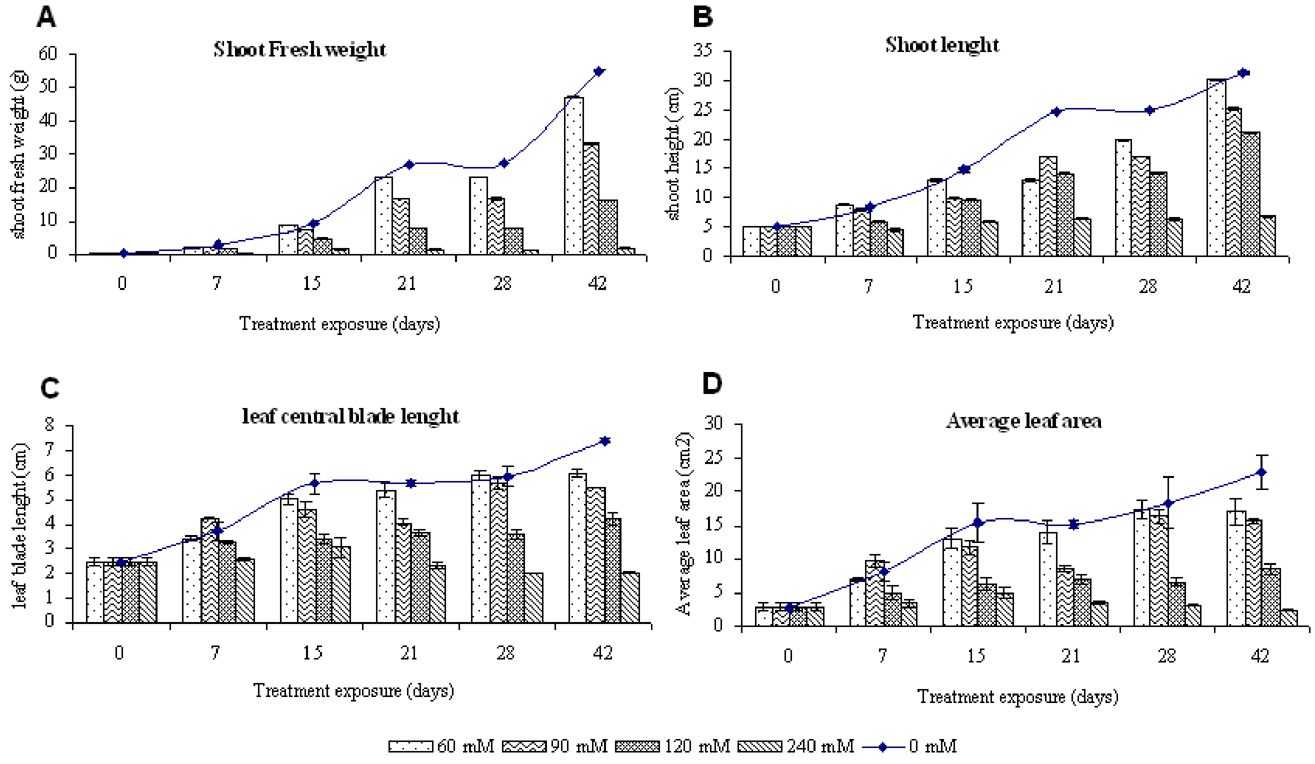


Figure 3

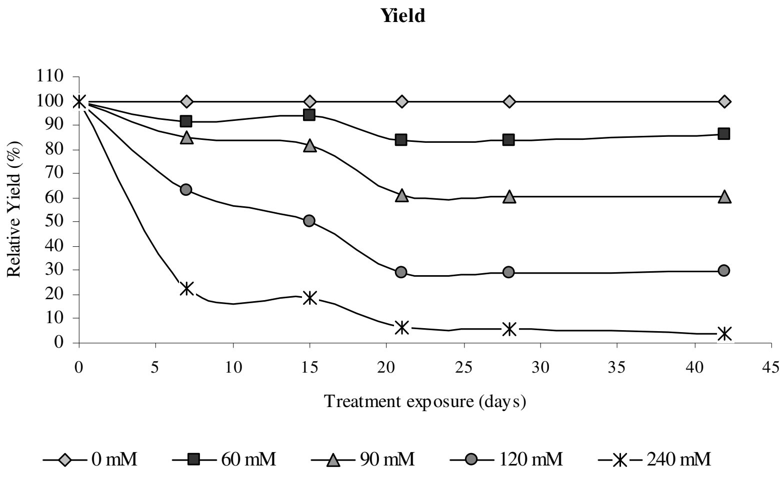


Figure 4

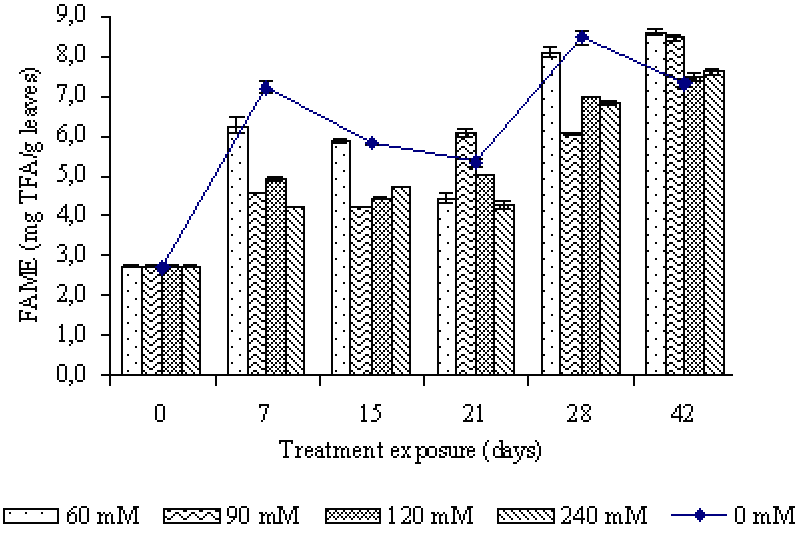
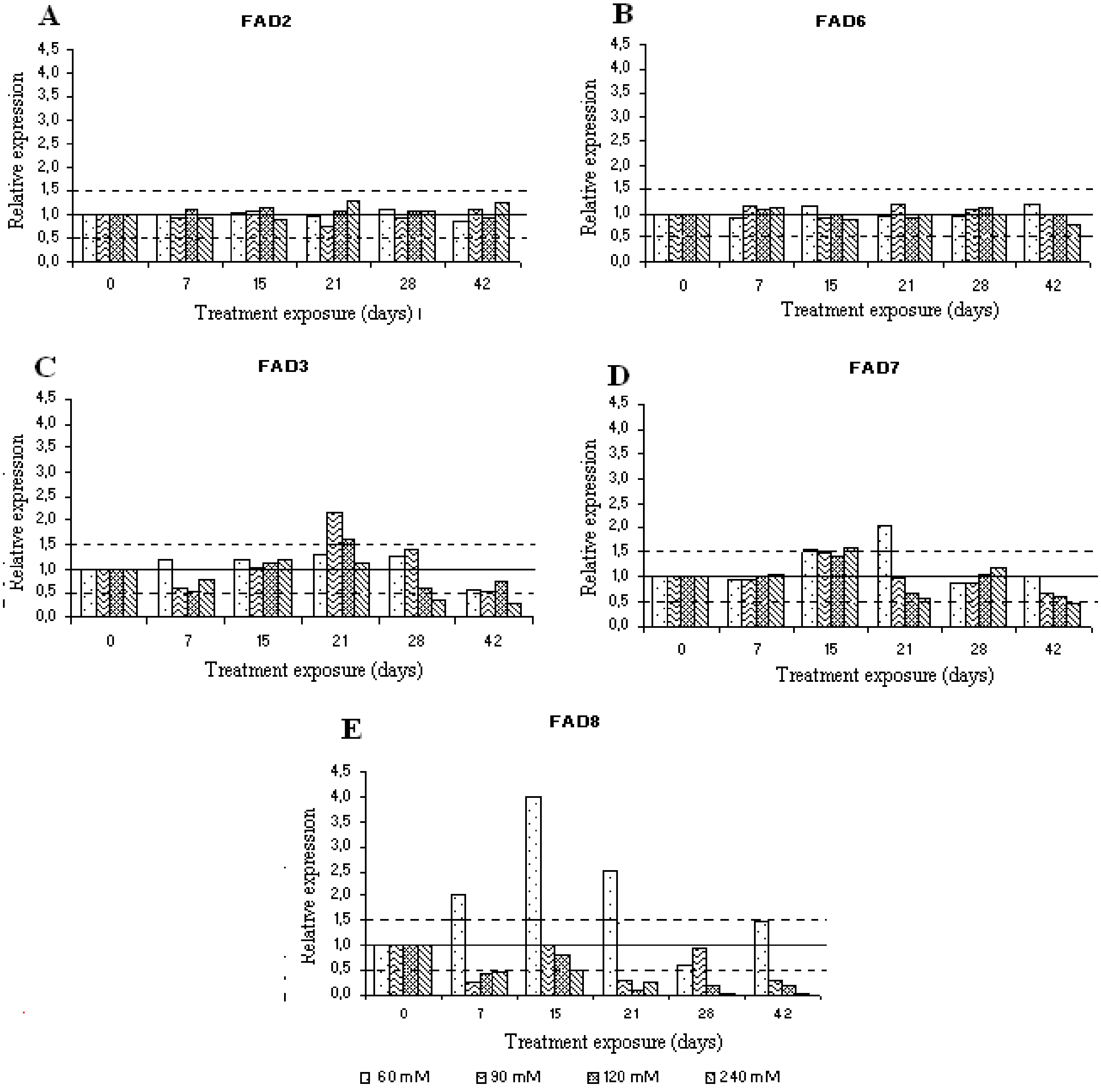

9,0
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
FAME (mg TFA/g leaves)
0
7
15
21
28
42
Treatment exposure (days)
60 mM
90 mM
120 mM
240 mM
0 mM

Figure 5


A
FAD2
B
FAD6
C
FAD3
D
FAD7
E
FAD8
Relative expression
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
7
15
21
28
42
Treatment exposure (days)
60 mM
90 mM
120 mM
240 mM

PAPER V

Establishment of in vitro plants, cell and tissue cultures from *Portulaca oleracea* for the production of polyunsaturated fatty acids

(Manuscript)

Establishment of in vitro plants, cell and tissue cultures from *Portulaca oleracea* for the production of polyunsaturated fatty acids

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Abstract

In vitro plants from *Portulaca oleracea* were established from seeds and grown on a hormone-free medium. *In vitro* purslane shots produced polyunsaturated fatty acids in concentrations of 1.57 mg LA/g FW (18% of total lipid), and 3.51mg/g FW linolenic acid (52 %) dry weight after growth for 30 days, which corresponded to 53% and 60% of linoleic and linolenic content, respectively, of *in vivo* plant shoots. Callus and cell suspension cultures could be initiated from aseptic leaf explants of *P. oleracea* seedlings. Chlorophyl containing friable callus were able to grow under dim illumination in the presence of an organic carbon source and retain their ability to produce polyunsaturated fatty acids (PUFA). These callus were then used for the establishment of a cell suspension culture. Fresh and dry weight measurements a 5-fold increase in biomass after four weeks of incubation. A partial differentiation of the cells into semi-differentiated green oval structures occurred during the incubation in liquid medium. Medium hormone composition affected the growth, and both chlorophyll and PUFA accumulation in these structures. No linolenic acid was produced in the green friable callus previously to the transference into liquid media. Morphological differentiation seems to be essential for linolenic acid production and culture establishment in liquid media. Therefore, several semi-differentiated as well as organized cell lines of *P. oleracea* have been developed. These cell lines will constitute a worthwhile starting point for the optimization of linoleic and linolenic biosynthesis in liquid media to the objective of producing polyunsaturated fatty acids under controlled and defined conditions in bioreactors.

Abbreviations: BA- 6 –benzylaminopurine; NAA-naphthalene acetic acid; K-Kinetin, 2,4-D - PUFA- polyunsaturated fatty acids; LA- linoleic acid; ALA-α-linolenic acid

1. Introduction

In plants, lipids play important roles in energy storage, membrane structure, biological activity, and surface coverings, in addition to some specialized functions such as light harvesting during photosynthesis. Unsaturated fatty acids are essential components required for normal cellular function, being involved in roles ranging from membrane fluidity to acting as signal molecules (Gil and Valivety, 1999; Broun *et al*., 1999). In particular, the class of fatty acids known as the polyunsaturated fatty acids (PUFAs) has attracted considerable interest as pharmaceutical and nutraceutical compounds (Broun *et al*., 1999; Horrobin, 1990).. PUFAs can be defined as fatty acids of 18 carbons or more in length, containing two or more double bonds. Many PUFAs are also essential fatty acids, being required in the diet for normal development in mammals that cannot synthesize the primary essential fatty acid-PUFA linoleic acid (18:2, *n-6*)(Broun, 1999). PUFA are important as structural components of membrane glycerolipids and as precursors to signaling molecules such as jasmonates in plants and eicosanoids in animals (Creelman and Mullet, 1997; Spector, 1999). Unsaturatated fatty acids in organisms are elongated and further desaturated to physiologically active substances such as prostaglandins or leukotrienes (Bajpai and Bajpai, 1993) Based on the potential need for PUFAs in human nutrition and medicine, searches for new sources of these compounds have been conducted.

Plant seeds and nuts are rich as sources of fatty acids. However because PUFA production in plants is strongly influenced by meteorological factors, plant cell culture offers an attractive alternative for stable production of PUFAs. Plant cell culture such as *Ribes rubrum* was reported to produce 59% of its total lipids as PUFAs when cultivated at 10 ºC (Hirano et al., 1997). Higher plants generally synthesize up to C18 fatty acids but not C20 and C22 fatty acids. It has been reported that common purslane (*Portulaca oleracea*) are able to synthesize long chain fatty acids such arachidonic (C20:0) and Behemic (C22:0) acids, but not its unsaturated derivates (Guil et al, 1996, Liu et al., 2000).

Common purslane is a member of the Portulacaceae, which consist of more than 120 species of often succulent herbs and shrubs, and is listed in the World Health Organization as one of the most used medicinal plants. *P. oleracea*, hereafter referred to as purslane, is widespread as a weed and has been ranked the eighth most common plant in the world. Purslane has a long history of use for human food, animal feed and medicinal proposesand has been widely used as a potherb in the Mediterranean, Central European and Asian countries.). The aerial parts are used medicinally for alleviating pain and swelling and as an antiseptic (Chan *et al*.., 2000). The dried herb can be boiled and it is made into a tea/soup in China (Cai *et al*.., 2004).This plant was reported to have neuropharmacological actions, wound healing activities and bronchodilatory effects (Malek, *et al*.., 2004; Parry *et al*.., 2003; Rashed *et al*.., 2003). The water extracts of *P. oleracea* show no cytotoxicity or genotoxicity, and have been certified safe for daily consumption as a vegetable (Yen, Chen, & Peng, 2001).

The high content in omega-3 fatty acids of *P. oleracea* (Kumamoto *et al*., 1990; Omara-Alwala *et al*., 1991; Simopoulos, 2004; Palaniswany *et al.,* 2001,Ezekwe *et al.*,1999;) is an important factor in the prevention of heart attacks and strengthening of the immune system (Simopoulos, 2004).The presence and concentration of α-linolenic acid in purslane may vary with the cultivar, geographic distribution, developmental stage and environmental factors (Liu *et al*.., 2000, 2002; Ezekwe *et al*.., 1999; Palaniswamy *et al*., 2001) The content of α-linolenic acid (ALA) in purslane also varies with the age and tissue-type, with leaves containing higher concentrations then the stems (Simopoulos *et al*.., 1992). ALA accounted for 60% of TFA in leaves, 30-40% in seeds and 10 to 25% in stems (Guil *et al*., 1996; Liu *et al.,* 2000). Except for linseed, in which ALA is 50-60% of the total fatty acids, most other oil crop seeds contain only 3 to 10 % ALA. So purslane constitutes an excellent vegetable source of ALA and it a good plant system to use for the development of cell lines for in vitro production of PUFAs. Considering that proliferation efficiency is a potential limiting factor in using plant cell culture for PUFA production, it is plausible to improve PUFA production by a better understanding on how PUFA biosynthesis is related to cell growth and/or cell differentiation, and by improving either of these items.

The main objective of this study is to establish *P. oleracea* cell culture lines for in vitro production of PUFA and investigate the effect of cell growth and/or cell differentiation in PUFA synthesis and accumulation *in P. oleracea* in vitro cell cultures.

2. Material and methods

2.1. Plant material and in vitro culture

P. oleracea seeds were acquired in a local shop. Under aseptic conditions, the seeds were immersed in 70% ethanol for 2 min and surface sterilized using 1% sodium hypochloride solution for 20 min, washed with sterile distilled water until reach a neutral pH, and germinated under standard methods on solidified tap water medium and on MS basal medium (Murashige and Skoog, 1962) containing 2% sucrose in the dark. The pH of the medium was adjusted to 5.8 prior to autoclaving for 20 min at 121 ºC. The cultures were maintained in 16-h photoperiod of 40 µmol $m^{-2}s^{-1}$ at 26 ± 2 ºC in an incubator (Termaks KBP 6395F, Bergen Norway). In vitro plants were maintained by periodic subdivision into an agar-solidified MS basal medium containing sucrose (30 g l^{-1}).

2.2. Callus initiation and determination of optimal hormone concentration for organogenesis

Callus of *P. oleracea* was developed from roots, stems and leaves from seedlings and in vitro grown plants. Plant material was cut into 2-3 mm-wide explants. Ten explants were placed on solid MS medium containing , thiamine HCL (0.1 mg l^{-1}), nicotinic acid (1.0 mg l^{-1}), pyridoxine HCl (1.0 mg l^{-1}), meso-inositol (100 mg l^{-1}) and sucrose (20 g l^{-1}), supplemented with different hormones (2,4D; naphthalene acetic acid [NAA], kinetin; 6 benzylaminopurine [BA]) and concentrations from 0 to 1.0 mg l^{-1}, adjusted to pH 5.7. The explants were incubated at 24 ± 2 ºC in 40 µmol $m^{-2}s^{-1}$ continuous light in a 16-h photoperiod or in the dark. Cultures were monitored weekly for callus development during a period of three weeks. The initiated calli were transferred to fresh media containing the same hormonal concentrations.

2.3. Initiation of suspension cultures and determination of growth rates

A cell suspension culture was prepared by transferring 0.5-1.0 g friable green callus to 250 ml flask containing 60 ml MS liquid media supplemented with 20 g l^{-1} sucrose and the hormone concentration which yielded good cell growth. The cultures were continuously shaken at 100 rpm and incubated at 24 ± 2 ºC under continuous light (30 µmol $m^{-2}s^{-1}$) in a 16-h photoperiod.
Growth rate of cell culture was measured by growing individually five 1.0 g samples of fresh cells in 250 ml erlenmeyer flasks flash containing 60 ml medium over a period of 8 weeks. The cultures in these samples were filtered thought cellulose filter paper under vacuum, fresh weight was determined, and the weighted cells were placed in an oven at 70 ºC overnight for dry weight determination.

2.4. Fatty acid isolation and determination

Fatty acids were extracted from leaves of green house plants (approximately 100 g fresh weight) and in vitro plants (approximately 20 g of fresh weight). Dried and powdered plant material (2 to 5 g) was use. The one-step extraction-methylation procedure hereby applied was based on the protocol referred by Vrinten *et al* (2005). 0.5 g of dried plant material was suspended in 2 ml of 3 N methanolic HCl supplemented with the internal standard, heneicasoenic acid (C21:0) in the concentration of 1.0 mg/ml, and incubated at 80ºC for 2 h. 1 ml of 0.9 % NaCl was added to stop the methylation reaction and the methylated fatty acids were extracted with 2x2 ml of hexane. For a better separation, the extract was centrifuged at 3000 g for 10 min. The hexane layer was transferred to a new tube and evaporated to dryness in a current of helium gas. The extract was resuspended in 1 ml of hexane for fatty acids analysis on a GC-MS.

The equipment used was an Agilent 6890 Series equipped with a column SGE 25QC2/BPX70 (25 m × 220 μm × 0.30 μm) and connected to a mass detector Agilent 5973 Network. The samples were injected with an automatic injector Agilent 7683 Series in the split less mode at 250 ºC. The oven was programmed to hold temperature at 130 ºC for 1 min, increase to 180 ºC at 25 ºC/min, and then and hold for 5 min to 230 ºC at 2.5 ºC/min. Helium was used as gas carrier with a velocity of 59 cm/s. The temperature of the ionization source and the quadruple were 230 ºC and 150 ºC, respectively. Fatty acid methyl esters were identified by comparing GC retention times with those of a mixture of standard fatty acids methyl esters (FAMEs) Mix C14-C22, (Supelco). Quantitative analysis of fatty acids were carried out by comparing their peak areas to that 0.5 mg internal standard, and calibrated with factors that were previously determined for each fatty acid.

Given to the relevance of plant cells and organ cultures as effective systems for natural products synthesis on a large scale, in vitro plants and several cell lines from *Portulaca oleracea* were established in this study in order to cultivate then for the controlled and standardized production of PUFAs in bioreactors.

3. Results and discussion

3.1. Portulaca oleracea in vitro and in vivo plants

Propagation of *P. oleracea* plants

Surface sterilized *P. oleracea* seeds produced normal seedlings with 100 % germination o MS basal medium. Initial germination of the seeds was achieved in a light-dark cycle (16:8 h) between the second and the fourth day of incubation at 24± 2 ºC. After 7-10 days the seedlings were 20-40 mm in height. Cultivation of 2-3 weeks on MS medium without phytohormones resulted in the generation of 2 to 3 nodes of the in the shoot of in vitro grown seedlings. Shoot apices with two to four leaves were isolated as primary explants and cultivated in MS medium supplemented with 20 g l^{-1} sucrose. Apical segments were used as initial explants because of their morphogenetic potential. After 20-30 days of culture multiple shoots were obtained directly from apical or auxiliary buds. The green house plants formed twines with faint red-colour stems whereas the stems in vitro plants were smaller with a bright wine-colour. The green house plants develop large golden-green oval leaves with a size of 2-3 cm long, and 1-2 cm width, whereas the leaves in the in-vitro plants were smaller up to 1.0 cm long and 0.8 cm wide and had a slight reddish coloration on the lower face and borders of the leaves.

Polyunsaturated fatty acid concentration in *P. oleracea* plants

For the detection of PUFAs in *P. oleracea* plants, a fatty acid profile was originated by GC-MS analysis from samples of plants cultures in the green-house plants (*in vivo*) as well as in aseptic in vitro cultures. In the in-vitro cultures, PUFAs composition was similar to the plants in the green house and in nature growing *in vivo* plants (Ezewke et al (2001). Palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) acids and traces of arachidic (C20:0) and behemic (C22:0). Polyunsaturated fatty acids fraction (C16:1, C18:1, C18:2; C18:3) corresponded to 65-76% of total fatty acids (TFA) in *in vitro* plants and 80-84 % TFA in *in vivo* plants. Lower concentration of linoleic and α-linolenic acids were detected in the in vitro plants (Fig. 1).

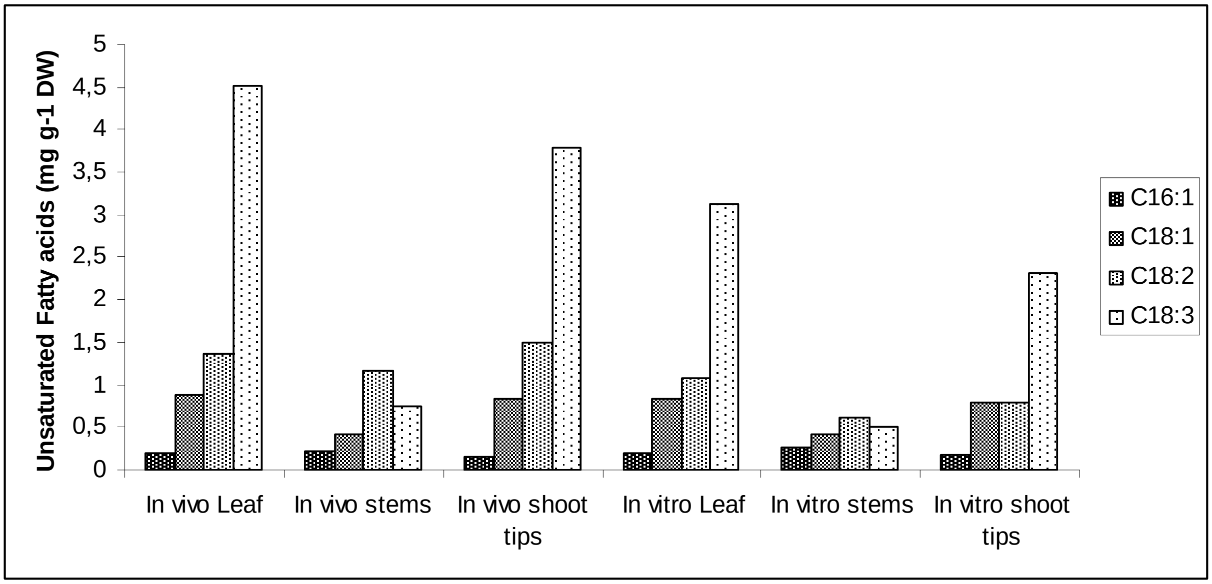

Figure 1.Unsaturated fatty acids concentration in different *P. oleracea* parts. C16:1 – Palmitoleic acid, C18:1- Oleic acid, C18:2 – Linoleic acid, C18:3 - α-Linolenic

A detailed analysis of fatty acid content of parts of the green house plant showed high concentrations of the linoleic (LA) and α-linolenic (ALA) acids (Fig 1). Linolenic acid concentration in the leaves, stems and shoots was in accordance to that found in literature. Guil *et al.* (1996) and Liu *et al.* (2000) examine the fatty acid content in *P. oleracea* tissues and reported that ALA accounted for 50 to 60% of TFA in leaves, 30-40% in seeds and 10 to 25% in stems. The concentration of linoleic and linolenic acids in the leaves of in vitro plants was about two 79 and 69 % of what of comparable leaves from the green house plant (Fig 1). In a similar way, the concentration of linoleic and α-linolenic acids in the stems and shoots of in vitro plants was about 53 % and 60-68% of what of comparable stem and shoot from the green house plant (Fig 1). Palmitoleic and oleic acids content was similar in the in vitro and in vivo plants, with a slight increase (10-15 %) in amount and relative proportion in the in-vitro leaves and stems. This data suggest that the leaves should be use as a source for callus induction, since higher amounts of linoleic and α-linolenic were found in this organ.

3.2. Portulaca oleracea cell and organs

Initiation of callus from young *P. oleracea* seedlings

Morphological and physiological differentiation of cell cultures can be initiated from callus and suspension cultures, or due to the initiation of organogenesis via different phytohormone concentration and/or ratios. Cut and wounded roots, stems and leaves were transferred to different media (MS supplemented with 2,4 D, NAA kinetin and BAP) to establish various cell culture lines from approximately 7 day-old seedlings. Callus initiation in the dark occurred two to three weeks of incubation. White and yellow friable calli were developed in leaves and root explants, whereas white and pinkish calli develop in the stem. These cultures were growing slow with a doubling time of about 4 weeks. After four to six weeks 6 different cell lines from leaves, roots and stems of *P. oleracea* were initiated. Callus initiation from leaves and stems cultured in light started sooner, after 2 weeks of incubation; depending on phytohormone concentration, green compact embryo-like structures or green friable calli were developed in leaf explants. The calli, originated from leaves and roots placed on MS salts with only kinetin (0-0.5 mg l^{-1}) or BA (0-1.0 mg l^{-1}) showed a compact growth (Table 1) originating compact green embryo-like structures (CGEC) whereas calli cultivated with NAA/BA and 2,4D/BA grew as friable, crumbling culture (Table 1). Most of these calli were soft and disaggregated to fine cultures with similar microscopic cell forms and aggregates when transferred into liquid media. When placed on MS salts with only NAA (0-0.5 mg l^{-1}) the leaf and stems explants grow roots, and in the case of stem explants, the whole plant was regenerated (Table 1). Stem explants, cultured on MS medium with both auxins and cytokinines supplementation originated aggregates of differentiated red leaf-like structures (RLE).

As indicated in Table 1, a mixture of auxins and cytokines seems to be essential for the development of friable callus. High NAA/BAP ratios induced the development of friable callus (WFC, GFC) and low NAA/BAP ratios resulted in partial tissue differentiation, with the development of GCEC and RLE .

Table 1: Effect of auxins (NAA, 2,4D) and cytokines (BA, kinetin) concentration on cultures developed from different *P. oleracea* explants

Explant	Root			Stem			Leaf		
NAA (mg l^{-1})	0	0.5	1.0	0	0.5	1.0	0	0.5	1.0

Light	0	0	R	R	R, gL	W	W	R	gf	R,gf
BA	0.5	gf	gs	gs	gf	gs, rl	gs, rL	gf	gs	gs
	1.0	gf	gf	gs	Gf	gf	gs	gf	gs	gs
kinetin	0	0	0	0	R	0	0	0	0	0
	0.5	Gf	gs	gf	R, rL	gs, rL	gf, rL	0	gf	Gs
	1.0	gf	gs	gf	R	0	gf	0	gf	gf
Dark	0	0	0	0	0	0	wf, rf	0	wf	wf
BA	0.5	0	ws	ws	0	ws	ws,rs	0	ws	ws
	1.0	0	ws	ws	0	rs	rs	0	ws	ws
Kinetin	0	0	0	0	0	0	0	0	0	0
	0.5	0	wf	ws	0	ys	rf	0	wf	ws
	1.0	0	wf	wf	0	Ys	bs	0	ws	ws
2,4-D (mg l^{-1})		0	0.5	1.0	0	0.5	1.0	0	0.5	1.0
Light	0	0	0	0	0	rL,gf	rL	0	wf,gf	wf,gf
BA	0.5	0	gf	gf	rL	rL,gf	rL,gs	gf	gs	gs, rL
	1.0	gf	gs	gs	rL	rL	rL	gf	gf	gf
kinetin	0	0	0	gf	0	0	0	0	0	0
	0.5	0	gf	0	0	0	0	0	0	gf
	1.0	0	gf	gf	0	rL	0	gf	gf	gf
Dark	0	0	0	0	0	0	0	0	0	0
BA	0.5	0	wf	bj	0	sw	sy	0	sw	sw
	1.0	0	0	0	0	sy	sb	0	sw	fw
Kinetin	0	0	0	0	0	0	sr	0	0	0
	0.5	0	wf	0	0	wrs	rs	0	0	0
	1.0	0	0	bf	0	wrs	rs	0	sw	0

w-white, g-green, y-yellow, r-red, b-brown, s-soft, f-firm, L-leaf regeneration, R-root regeneration, H- hole plant regeneration

The initiation of callus cultures was faster in the dark than under illumination. The induction of cell cultures from root explants is promoted in the dark, which is due the presence of meristem cells and the absence of chloroplast in these cells. An expected morphological differentiation from different explants from *P. oleracea* in vitro seedlings was found only in cultures kept in light. Most of the cell cultures obtained from stem explants during the differentiation assay indicated a morphological differentiation into leaf-like structures; however some cultures develop soft white/green aggregates. Although we observed the development of soft friable callus in medium supplemented with combined mixtures of both auxins (NAA and 2,4-D) and both cytokines (BA and Kinetin) tested, the medium with NAA/BA combinations was more prolific in friable callus development, particularly from leaf explants, and so this two phytohormones were chosen for further optimization. For others, the differentiation noticed was on the one hand a morphological development in terms of rooty cultures as well as of slow growing firm tissue resulting from root and leaf explants. Soft white/green aggregates were also developed from leaf explants kept in dark/light conditions. Physiological differentiation process was also observed, indicated by the production of chlorophyll in greenish (g) betalains in red (r) and phenol in brownish (b) cultures (Table 1).

3.3. Initiation of *P. oleracea* liquid cell cultures

To evaluate witch tissues would be more suitable for in vitro PUFAs production; we analyzed the fatty acid composition of the four main structures formed in solid media by GC-MS analysis: white friable calli (WFC), green friable calli (GFC), green compact embryo-like callus (GCEC) and red-leaf efflorescence (RLE). Fatty acid profile was similar between the studied structures. Palmitic (C16:0), palmotoleic (C16:1), Stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) acids and traces of arachidic (C20:0) and behemic (C22:0) were detected in the cell cultures (Fig. 2). Polyunsaturated fatty acids fraction (C16:1, C18:1, C18:2; C18:3) corresponded to 75-80% of total fatty acids (TFA), with the exception of WFC and GFC in which palmitoleic (C16:1) and oleic (C18:1) acids constituted the main fraction of PUFA (60 and 25 % TFA, respectively) and low levels of linoleic acid (inferior to 6 %) were present. α-linolenic acid was not detected or was detected in trace amounts in this cell cultures (Fig. 2). GFC and GCEC profiles were similar, with a higher accumulation of linoleic acid (30% TFA) than α-linolenic (10-15 %TFA). This result may indicate that tissue differentiation and illumination are necessary for linolenic acid production. Due to these results we access that

WFC are not fit to be used for the production of PUFAs in suspension cell cultures, at least not on these conditions. It makes sense, since some of the desaturase genes are localized in the chloroplast membranes, so it is possible that tissue morphological and physiological differentiation is necessary for PUFA biosynthesis.

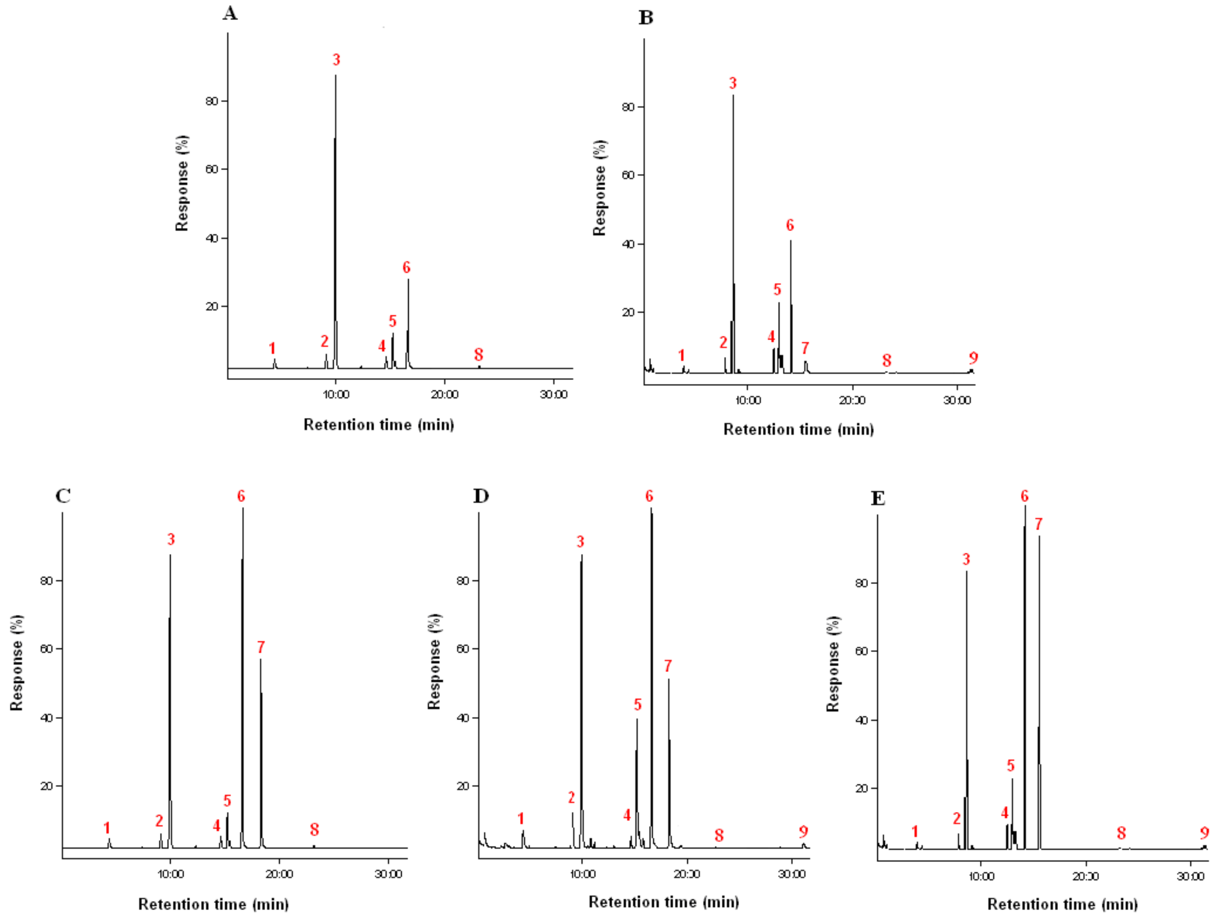

Figure 2 – Fatty acid profiles of *P. oleracea* cell cultures developed in solid and liquid MS medium. WFC (A) and GFC (B) cultured in MS solid medium supplemented with NAA/BA phytohormones. Four week old GCC (C), GCEC (D) and RLE cultures maintained in MS liquid medium supplemented with NAA/BA phytohormones.

It is well known that morphological differentiation and biochemical processes are often linked in plant cell cultures. For many species, the synthetic capacity of dedifferentiated cell was lower than that of the fully differentiated tissue, both quantitatively and qualitatively. Chemical gradient in differentiated tissue complexes or callus aggregates encourage the synthesis of secondary metabolites (Dörnenburg and Knorr, 1995). To access if the other cell culture could be used for PUFAs production in biofermentors, 1-2 g of each of these structures was transferred to liquid MS medium supplemented with the same phytohormones where they were developed, and cultured for 4 weeks. No significant changes to the tissue morphology or fatty acid profile were observed in the cell cultures cultivated in liquid medium, compared to the original structures maintained in solid medium, with the exception of the GFC, which formed oval green aggregates with a semi-differentiated structure (GCC) that were able to accumulate linolenic acid. Although, the highest amount of linolenic acid was present in RLE (with equivalent amounts of LA and ALA), as it was previously observed in the solid medium cultures, maintenance in liquid culture was difficult due to the large aggregate structures formed that accumulated in the bottom of the flask, which would make impossible to keep this culture in a biofermentator.

A literature survey indicated a close correlation between morphogenesis and organization of tissue for the production of other secondary metabolites, such as cyclic peptides (Svangård et al., 2003; Trabi and Craik, 2004) and PUFAs (Chiou et al. 2001).

Results from the fatty acid analysis indicate the need for a morphological differentiation of *P. oleracea* cell cultures because of the correlated metabolic differentiation. This was pursued via variation of phytohormones concentration and ratio. Due the rapid proliferation and biomass production shown by GFC in liquid culture, we decided to use them as starting point for fatty acid production, and we tested different NAA/BAP ratios in medium in order to optimize linolenic synthesis and accumulation. 1.0 g of GFC were transferred to liquid MS medium supplemented with NAA/BA ratios of 2.1 (CIM1), 1:1 (CIM2), 1.2 (CIM3) and 1:10 (CIM4). Cell differentiation has been achieved in liquid cultures from GFC under 16:8 h photoperiod.

Table 2 – Characteristics off different *P. oleracea* liquid cell cultures established from leaf explants during active growth phases up to 60 days after inoculation.

Medium	Inocculum (mg DW l^{-1})	Water content (%)	Biomass (g DW l^{-1})	Compact callus average size* (cm)	Compact callus color*
CIM1	50	95.3	3.1	0.1-0.2	White-yellow
CIM2	50	96.2	7.4	0.3-0.5	Light green

CIM3	50	97.8	8.5	0.7-1.0	green
CIM4	50	98.0	13.0	1.0-1.3	Dark-green

* See Figure 3

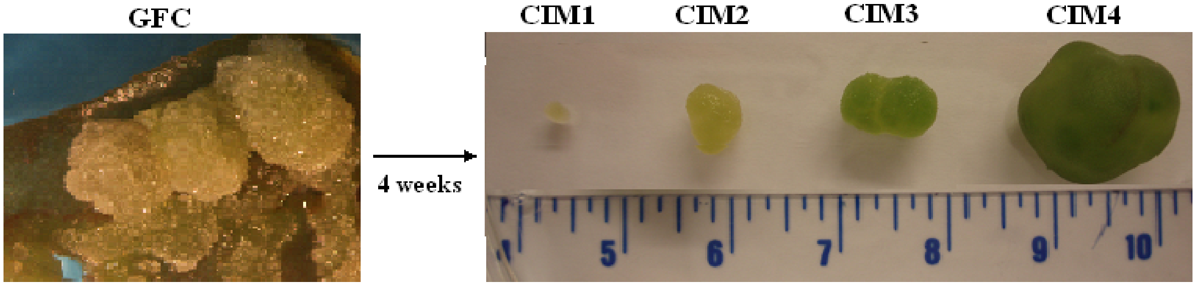

Figure 3. Four week old cultures with different NAA/BAP proportions: CIM1 (2:1), CIM2 (1:1) CIM3 (2:1) and CIM4 (1:10). GFC – Green friable callus

Morphological occurred in terms of the development of oval compact structures with yellow (Y) to dark-green color with a diameter variation of 0.1 to 1.2 cm were developed. GFC cultured in medium supplemented with the lowest NAA/BAP ratio (1:10) showed the best biomass accumulation in the same incubation period, forming large structures with an average diameter of 1.2 cm with a dark-green coloration. As the NAA proportion in medium decreased, the size and intensity of the green colour decreased. Physiological differentiation process was also observed, indicated by the production of betacyanins in yellow (Y) and chlorophyll in greenish (g). Phenol synthesis and accumulation occurred at later stages of incubation (9 weeks), when some of the cultures started to turned from bright green to a brownish color (b), maybe as a sign of stress (Table 2). This data suggest that the hormone proportion in medium affects tissue differentiation and growth, with higher auxins values negatively affecting the dimension of the formed structures. In table 3 are represented the unsaturated fatty acids accumulation patterns of *P. oleracea* cells cultured in MS liquid medium supplemented with different NAA/BAP concentrations.

Sample	Harvest (week)	Concentration(mg FAME/g tissue DW)						Concentration (% in oil)					Ins/sat	ALA/LA
		C16:0	C18:0	C18:1	C18:2	C18:3	Total	C16:0	C18:0	C18:1	C18:2	C18:3		
CIM1	4	8,78	0,03	2,09	6,78	7,35	25,03	35,1	0,1	8,3	27,1	29,4	1,8	1,1
	5	9,22	0,24	2,31	7,14	8,09	27,00	34,1	0,9	8,6	26,4	30,0	1,9	1,1
	6	10,22	0,41	2,56	7,87	8,37	29,44	34,7	1,4	8,7	26,7	28,4	1,8	1,1
	7	4,70	0,20	0,42	4,04	4,27	13,64	34,5	1,5	3,1	29,6	31,3	1,8	1,1
	8	8,33	0,18	0,96	6,97	5,80	22,23	37,5	0,8	4,3	31,4	26,1	1,6	0,8
	9	6,89	0,46	0,95	4,85	3,08	16,22	42,4	2,8	5,8	29,9	19,0	1,2	0,6
CIM2	4	9,10	0,59	2,00	6,50	7,80	25,99	35,0	2,3	7,7	25,0	30,0	1,7	1,2
	5	9,08	0,56	2,01	6,48	7,81	25,94	35,0	2,2	7,7	25,0	30,1	1,7	1,2
	6	9,10	0,59	2,00	6,50	7,80	25,99	35,0	2,3	7,7	25,0	30,0	1,7	1,2
	7	9,24	0,48	1,55	6,51	9,40	27,18	34,0	1,8	5,7	24,0	34,6	1,8	1,4
	8	6,65	0,19	0,66	4,28	4,03	15,81	42,0	1,2	4,2	27,1	25,5	1,3	0,9
	9	7,40	0,38	1,02	4,88	3,96	17,64	42,0	2,1	5,8	27,6	22,4	1,3	0,8
CIM3	4	9,26	0,43	1,77	8,30	9,24	29,00	31,9	1,5	6,1	28,6	31,9	2,0	1,1
	5	9,10	0,40	1,48	8,00	7,85	26,83	33,9	1,5	5,5	29,8	29,3	1,8	1,0
	6	8,65	0,37	0,91	7,10	6,96	23,99	36,0	1,6	3,8	29,6	29,0	1,7	1,0
	7	5,28	0,27	0,44	3,68	4,18	13,85	38,1	1,9	3,2	26,6	30,2	1,5	1,1
	8	5,17	0,52	0,69	3,70	2,48	12,56	41,2	4,2	5,5	29,4	19,8	1,2	0,7
	9	4,77	0,38	0,38	3,71	0,90	10,13	47,0	3,7	3,8	36,6	8,9	1,0	0,2

CIM4	4	5,83	0,42	0,46	5,96	3,54	16,21	42,7	3,1	3,4	43,7	25,9	1,6	0,6
	5	5,48	0,58	0,54	6,21	3,35	16,16	24,7	2,6	2,4	27,9	15,1	1,7	0,5
	6	5,28	0,77	0,71	6,60	3,14	16,50	32,6	4,7	4,4	40,7	19,4	1,7	0,5
	7	6,61	0,60	0,68	7,36	3,34	18,59	25,4	2,3	2,6	28,3	12,8	1,6	0,5
	8	5,20	0,55	0,52	4,57	1,22	12,06	20,0	2,1	2,0	17,6	4,7	1,1	0,3
	9	4,89	0,27	0,65	2,92	1,70	10,43	18,8	1,0	2,5	11,2	6,5	1,0	0,6

Table 3 – Fatty acid concentration, in dry weight basis, of *P. oleracea* cultures maintained in liquid medium supplemented with different NAA/BAP proportions.: CIM1 (2:1) , CIM2 (1:1), CIM3 (1:2), and CIM4 (1:10).

C16:0 – palmitic acid, C18:0 – stearic acid, C18:1- Oleic acid, C18:2 – Linoleic acid, and C18:3 - α-Linolenic acid

According to this data, the medium that appear to be better suited for polyunsaturated in vitro biosynthesis is CIM2, and harvest should be done between 6 and 8 weeks of incubation, where higher amounts of α-linolenic acid are accumulated. Although the α-linolenic accumulation is superior in CIM1, for shorter periods of incubation (5 to 6 weeks, Fig 3), the biomass production was low (Fig. 3), so higher amounts of starter culture would be necessary to produce significant amounts of the target products. In the other hand, both CIM2 and CIM3 have similar linoleic and α-linolenic acids content at the same incubation period, and since CIM2 uses less amount of cytokines in the media, its use would be preferable from a commercial point of view. After selecting and finding the adequate growth conditions for the different cell lines, these cultures can be used for polyunsaturated acids production in bioreactors as has been reported for fine suspension cell cultures.

4. Conclusions

The data presented here illustrated for the first time the initiation and establishment of in vitro plant cultures of *P. oleracea* able to accumulate high levels of PUFAs. The results cleary showed that PUFA accumulation is dependent on the type of culture used, and seems to be correlated with morphogenesis. Leaves and shoots were characterized by the highest content of PUFAs, showing that the biosynthesis and/or accumulation of these compounds is a also related to the tissue differentiation.

Plant cell cultures growing in liquid medium are preferable for the production of useful metabolites in large scale bioreactors (Zhong, 2001). Due to homogenous mass transport growth rates of the suspension cultures are in general higher. We were not able to obtain suspension cell cultures from *P. oleracea* in the tested conditions since morphologic differentiation occurred when the friable calli were transferred from solid to liquid medium. Furthermore, morphological and physiological differentiation of callus was manipulated by phytohormone concentration. Each or a different combination of parameters, such as the origin of explants used for the establishment of cell cultures, the cellular and/or tissue differentiation status, stress factors, and culture conditions can be used to increase the yield of target products in cultured plant cells and tissues (Dörnenburg and Knorr, 1995; Yeoman and Yeoman, 1996). The establishment of differentiated cultures as well as special strategies will be effective tools to use organized as well as undifferentiated cell lines from *P. oleracea* for PUFA production. In order to induce and optimize production for bioreactor technology, key factors such as medium composition, phytohormone concentrations, stress physiology and influence of light must be studied in more detail. Therefore, favorable conditions will be determined and experimental designs are underway to study the effect of several process parameters. Gathering of all this information will allow improvements in the cultures of *P. oleracea* for micro propagation, metabolic studies, and for PUFAs production in bioreactors.

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