‘Studies on the potential of alternative oxidase (AOX) as a functional marker candidate for efficient adventitious rooting in olive (*Olea europaea* L.)’

Keywords: alternative oxidase, adventitious rooting, salicyl-hydroxamic acid, polymorphism, phenylpropanoid, lignin, olive propagation, Functional Marker.

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‘Estudos sobre o potencial da oxidase alternativa (AOX) como candidato a marcador funcional para a eficiência no enraizamento adventício de oliveira (*Olea europaea* L.)’

Resumo

O envolvimento e a importância da oxidase alternativa (AOX) na resposta das plantas a diversas condições de *stress* têm vindo a ser demonstrado. Tendo em conta esse conhecimento, colocou-se a seguinte hipótese: “o enraizamento adventício (EA) é a uma reação da planta ao *stress* abiótico no qual a AOX tem um papel determinante”. Com a finalidade de testar a hipótese e avaliar o potencial da AOX para o desenvolvimento de um marcador funcional (MF), relacionado com a eficiência no enraizamento adventício (EA) em *Olea europaea* L., quatro genes *AOX* foram isolados (*OeAOX1a*, *OeAOX1b*, *OeAOX1c* e *OeAOX2*). O envolvimento da AOX na resposta fisiológica ao EA foi estudado em dois sistemas distintos, num utilizando estacas semi-lenhosas e noutro microestacas. As cultivares de oliveira, ‘Galega vulgar’, recalcitrante ao enraizamento por estacaria semilenhosa e ‘Cobrançosa’, de fácil enraizamento por este processo, foram utilizadas nos ensaios. Os resultados obtidos em ambos os sistemas mostraram que o EA é significativamente reduzido pelo tratamento com um inibidor da AOX, o ácido salicilhidroxâmico (SHAM), enquanto que tratamentos com peróxido de hidrogénio ou piruvato, ambos indutores da AOX, resultaram num aumento das taxas de EA. Resultados similares foram obtidos de microestcas de ‘Galega vulgar’. O SHAM, reduziu significativamente o EA, resultado que foi confirmado por análises histológicas, porém não inibiu a formação de *calli*. As análises de metabólitos mostraram uma redução significativa na quantidade de fenilpropanóides e de lignina após o tratamento com SHAM, indicando que a atividade da AOX durante o EA estará associada ao metabolismo de adaptação desses metabólitos. O estudo da variabilidade nos genes *AOX*, realizado ao nível do ADN e ARN, centrou-se no gene *OeAOX2*. A maior frequência de polimorfismos foi identificada na extremidade 3'UTR. Diferenças no nível de acumulação de transcrito durante o EA foram observadas apenas quando se utilizou um primer *reverse* localizado na 3'UTR, o que indica a ocorrência de uma modificação pos-

transcripcional. Em suma, os resultados obtidos permitem considerar a AOX como enzima chave no processo de reprogramação celular associado ao EA. Em suma, os resultados obtidos permitem considerar a AOX como enzima chave no processo de reprogramação celular associado ao EA. Os resultados desta tese são encorajadores e abrem uma linha de investigação promissora com um grande potencial de aplicação que deverá ser considerado a nível regional/nacional/internacional.

‘Studies on the potential of alternative oxidase (AOX) as a functional marker candidate for efficient adventitious rooting in olive (*Olea europaea* L.)’

Abstract

It has been suggested that alternative oxidase (AOX) plays an important role during plant stress responses. In this thesis the hypothesis is that root induction is a plant cell reaction linked to abiotic stress and that the activity of stress-induced AOX is important for adventitious rooting (AR). In order to investigate *AOX* as a functional marker (FM) to assist AR efficiency in *Olea europaea* L., four *AOX* gene members (*OeAOX1a*, *OeAOX1b*, *OeAOX1c* e *OeAOX2*) were isolated from ‘Galega vulgar’, a cultivar with low AR efficiency. The involvement of AOX in AR physiological responses was studied in two different system, using a semi-hardwood cuttings and another microcuttings. The olive cultivars 'Galega Vulgar', recalcitrant rooting by semi-hardwood and 'Cobrançosa', easy to root for this process were used in the tests. Rooting was significantly reduced by treatment with salicylhydroxamic acid (SHAM), whereas treatment with hydrogen peroxide or pyruvate, increased the degree of rooting. A similar approach was adopted using microshoots of the cultivar ‘Galega vulgar’. SHAM significantly reduced AR, which was confirmed by histological analysis, but failed to exhibit any effect on the preceding calli stage. Metabolite analyses showed that the amounts of phenylpropanoids and lignin were significantly reduced following SHAM treatment, indicating that the influence of AOX activity on root formation was associated with adaptive phenylpropanoid- and lignin- metabolism. A detailed study of DNA and RNA level was carried out on *OeAOX2*. 3’-UTR was the most important source for polymorphisms. Differential transcript accumulation of *OeAOX2* during AR induction was observed when using primers in the 3’UTR indicating posttranscriptional modification. In summary, the results obtained allow to consider AOX as a key enzyme in cellular reprogramming associated with the EA process. The results of this thesis are encouraging and open a promising line of research with a great potential for application to be considered at regional / national / international level.

Abbreviations

ABA - Abscisic acid

ACC1 - aminocyclopropane-1-carboxylic acid

ADN – deoxyribonucleic acid

AFLPs - amplified fragment length polymorphisms

AluI - *Arthrobacter luteus*

AOX - Alternative oxidase

APA - alternative polyadenylation

ARF - Adventitious root formation

ARN - ribonucleic acid

ATP - adenosine triphosphate

bp - base pair

Bsp - *Bacillus species* RFL 143

Cdna - complementary DNA

COX - cytochrome oxidase

CS - cleavage site

Cyt - cytochrome

DEPC - diethyl pyrocarbonate

DMSO - dimethyl sulfoxide

DNA - desoxyribonucleic acid

dNTPs - deoxyribonucleotide triphosphate

DOP - Protected Denomination Origins

DTT - dithiothreitol

EcoRI - *Escherichia coli* RI

EtBr - ethidium bromide

ETCs - Electron transport chains

FAA - formaldehyde : acetic acid : 70% (v/v): ethanol

FAA - formaldehyde: acetic acid : ethanol (alcohol)

FM - Functional marker

FUEs - far upstream elements

gDNA - genomic DNA

H_2O_2 - Hydrogen Peroxide

HPyF3I - *Helicobacter pylori* RFL3

IAA - indole-3-acetic acid

IBA - auxin indole-3-butyric acid

ILP - intron length polymorphism

InDels - insertion/deletions

KCN - Potassium cyanide

(LA)-PCR - long and accurate- polymerase chain reaction

LSD - Least Significant Difference

MFE - Minimal free energy in kcal/mol

MgCl2 - Magnesium chloride

miRNA - micro RNA

mRNA - messenger ribonucleic acid

NaOH - Sodium hydroxide

$(NH_4)_2SO_4$ - Ammonium Sulphate

NMD - nonsense-mediated RNA decay

NO - nitric oxide

non-synonymous single nucleotide polymorphisms (nsSNPs)

NUEs - near upstream elements

OeAOX - *Olea europaea* L. *AOX* genes

OM médium - olive culture medium

ORF - Open Reading Frame

PAS - poly(A) site

PCR - polymerase chain reaction

POX - peroxidases

PPO - polyphenol oxidases

3'-RACE 3'- Rapid Amplification cDNA Ends

5'-RACE5' - Rapid Amplification cDNA Ends

RFLPs - restriction fragment length polymorphisms

RNA - ribonucleic acid

ROS - reactive oxygen species

SHAM - salicylhydroxamic acid

SNPs - single-nucleotide polymorphisms

SQ-RT-PCR - Semi-quantitative RT-PCR

SSRs - simple sequence repeats

TGA - thioglycolic acid

3'UTR - 3'-untranslated regions

Nucleotide Bases

A - Adenine

C - Cytosine

G - Guanine

T - Thymine

M - Amino (A or C)

N - Any nucleotide (A, C, G or T)

R - Purine (A or G)

S - Strong (G or C)

Y - Pyrimide (C or T)

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Chapter 2 Isolation and Characterization of AOX genes in *Olea europaea* L.

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Fig 14. Dendrogram describing the relationship among AOX1 proteins from higher plants, including the two complete sequences of *O.europaea* (*OeAOX1a* and *OeAOX1c*). The alignment was bootstrapped with 1000 replicates by the Neigbor-Joining method using the MEGA 4 software. The fungus AOX *Neurospora crassa* was used as outgroup. The scale bar indicates the relative amount of change along branches.

Chapter 3 Physiological responses and gene diversity indicate olive alternative oxidase (AOX) as a potential source for markers involved in efficient adventitious rooting induction

Fig. 1. Structure of the partial *OeAOX2* gene from *O. europaea* cv. Galega vulgar.

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Fig. 5. Alignment of the translated amino acidic sequences obtained from the three genotypes of *O. europaea*. The underlined sequence corresponds to the peptide sequence of exon 4, all the other peptide sequences correspond to exon 3 in reference to *A. thaliana*. Amino acid residues differing are shown on a black background, deletions are shown by minus signs. Sites of translation stop are shown by points and are related with a *nonsense* mutation. In black boxes are three regions defined by Berthold et al. (2000) as highly conserved in AOX. The di-iron site positions are indicated by filled triangles. Asterisks indicate residues found to confer resistance to the AOX inhibitor salicylhydroxamic acid (Berthold 1998). In grey boxes are two structural elements proposed to influence AOX regulatory behavior (region 3 and 4 proposed by Crichton et al. 2005), the residues potentially involved in regulation of AOX activity are indicated by filled circles. Helical regions that are assumed to be involved in the formation of a hydroxo-bridged binuclear iron center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown by two-headed arrows above the amino acid sequences. Possible membrane-binding domains center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown with line above the amino acid sequences. The peptide sequences presented in this figure refer to the ORF translation and the sequences given in table 2.

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Chapter 4 Adventitious root formation in olive (*Oleae europaea* L.) microshoots: anatomical studies

Fig. 1. Sections of the stem basal region, at root origin, 0-30days after IBA root inductive treatment. Panel A, anatomical structure of the stem-base before root-induction treatment, showing a vascular bundle; Pi, pith; Co, cortex; Ep, epidermis; Ph, phloem; X, xylem. Panel B, transverse section near the stem-base at 4 days. Cells in the cortex reacquire meristematic characteristics, presenting a dense cytoplasm with large nuclei and visible nucleoli (arrows); Ep, Epidermis; Co, Cortex. Panel C, first cell divisions (Cd) at 6 days, leading to calli formation. Magnification of the circular region is presented in Panel C*. Xylem tracheids (arrow) are also visible. Panels D and E, stem sections after 10 days on rooting medium showing two meristemoid structures (Me) in the upper phloem/cortex in Panel D and in the sub-epidermal region in Panel E. Magnifications of the circular regions are presented in Panels D* and E* respectively. Panel F, morphogenic root fields (Rf) developing from sub-epidermal cells 14 days after root induction treatment. Magnification of the circular region is presented in Panel F*. Panels G and H, root primordia (Rp) at different developmental stages, 22 days after root induction treatment. The root caps (Rc) and differentiated the vascular system (Vs) can be observed.

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Chapter 5 Involvement of alternative oxidadse (AOX) in adventitious rooting of *Olea europaea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism

Fig. 1. Inhibitory effect of SHAM on *in vitro* rooting of *Olea europaea L.* Root inhibition rate was positively correlated to SHAM concentration. IBA was applied for 10 s in a concentration of 14.7 mM.

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Fig. 4. Trends in total phenolic content changes in microshoots during the process of IBA-induced adventitious rooting process. Total phenolic content was expressed in milligrams of gallic acid equivalent per gram fresh mass. Values represent the average of three measurements ± SD (three biological samples each consisting of the bulked basal parts of 40 microshoots).

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codon. The intron is complete and consists of 499 bp. In the 3'-UTR region 159 bp downstream of the stop codon were analyzed. (*Cv.* corresponds to the cultivars; *M* corresponds to the material used for the gene isolation; *bc* corresponds to the number of sequences identified after *OeAOX2* cloning and sequencing).

Table 3. Computational prediction of intronic miRNA precursors in *OeAOX2* of three *Olea europaea* cultivars: 'Galega vulgar' (Gal), 'Cobrançosa' (Cob) and 'Picual' (Pic). *bp*: length of the pre-miRNA sequence in bp, *MFE*: minimal free energy in kcal/mol, *Prob*: probability to be a real pre-miRNA sequence.

Table 4. Alternative polyadenylation of the *OeAOX2* transcripts ('Galega vulgar') detected by 3'-RACE experiments. The length of the 3'-UTR in bases, the number and percentage of clones detected for each 3'-UTR size and the nomenclature of the sequence are shown.

Table 5. miRNA target sites predicted at the 3'-UTR region of the sequence Gal_3 (software miRanda v3.0).

Chapter 4 Adventitious root formation in olive (*Oleae europaea* L.) microshoots: anatomical studies

Table 1. Dehydrating solutions (values to prepare 100 ml of each solution)

Chapter 5 Involvement of alternative oxidadse (AOX) in adventitious rooting of *Olea europaea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism

Table 1: Genes and respective primers used for SQ-RT-PCR.

Thesis publications

The present work is based on the following manuscripts:

Macedo ES, Cardoso HG, Hernández A, Peixe AA, Polidoros A, Ferreira A, Cordeiro A, Arnholdt-Schmitt B (2009) Physiological responses and gene diversity indicate olive alternative oxidase (AOX) as a potential source for markers involved in efficient adventitious rooting induction. Physiologia Plantarum 137: 532-552

Macedo ES, Sircar D, Cardoso HG, Peixe A, Arnholdt-Schmitt B (2012) Involvement of alternative oxidase (AOX) in adventitious rooting of *Olea europaea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism. Plant Cell Reports 31(9): 1581-90.

Macedo ES, Vieira CM, Carrizo D, Porfírio S, Hegewald H, Arnholdt-Schmitt B, Peixe A (2013) Adventitious root formation in olive (*Olea europaea* L.) microshoots. Anatomical evalution and associated biochemical changes in peroxidase and polyphenol oxidase activities (Journal of Horticultural Science & Biotechnology 88 (1) 53-59.

Others publications

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Table of Contents

Chapter 1 Introduction

1.1. *Olea europaea*- Taxonomic position

Olive is a member of the *Oleaceae* family, which, among 29 other genera, includes *the* genus *Olea* (Martins and Sibbett 2005). According to Rugini et al. (2011) the genus Olea is split into three subgenera, *Tetrapilus, Paniculatae and Olea* (cultivated olive and its wild relatives). The subgenus *Olea* is divided into two sections: *Ligustroides*, with about 10 species and *Olea,* with only one species. The section *Olea* includes the complex of *Olea europaea* L., the Mediterranean olive tree, the only species cultivated for oil extraction and consumption. Also according to Rugini et al. (2011), *Olea europaea* accounts more than 1,000 varieties, although many of these might be just different landraces stemmed from the same original genetic stock and originating from mutations and natural hybridizations.

1.2. Olive Origin and Domestication

Olive-tree cultivation began in the eastern Mediterranean around 3000 BC, the first olive groves being established in Palestine and coastal parts of Syria and Turkey (De Candolle, 1883 cited by Tubeileh et al. 2004); it spread to Italy in around 1000 BC and extended westwards to Spain during the era of the Greco-Roman empire and later as a result of Arab-Islamic influence (Tubeileh et al. 2004). But numerous studies have confirmed that the olive has been present in the Mediterranean Basin for several thousands of years before its domestication, particularly in the Middle East (Rugini et al. 2011). The olive was probably domesticated in the Jordan River valley c. 5700–5500 BC (Zohary and Spiegel-Roy 1975), while according to Liphschitz et al. (1991) domestication occurred during the Bronze Age (5200 BC). There are many theories about the genetic origin of the cultivated olive but it is now generally accepted that the cultivated olive evolved from the *oleaster* or "wild olive" (*Olea europaea* var. *oleaster*), which shows a high degree of genetic variability and many characteristics that are similar to the cultivated olive (*Olea europaea* L.).

Individual *oleaster* trees showing superior performance in terms of size and/or oil content of the fruit were selected empirically and propagated vegetatively as clones, using cuttings that were planted directly or grafted onto indigenous oleasters. Olive varieties were then developed and distributed during successive human migrations throughout the Mediterranean basin, from east to west, although the selection of olive varieties was multilocal and originally included western plant material (Rugini et al. 2011).

1.3. Some generic aspects of *Olea europaea* L. botany

1.3.1. External morphology of the olive tree

Olive plants are evergreen trees with greyish-green foliage and the size of cultivated plants range from 4 to 8 metres in height (Rapoport 1998). It is a long-lived tree and a new dating method recently developed by the Portuguese University of Trás-os-Montes e Alto-Douro (UTAD) has enabled the identification of individuals in Portugal which are over 2,000 years old. The trunk is cylindrical, twisted and sometimes has characteristic knot-like swellings, it can measure more than two metres in diameter and presents a light gray bark. Olive wood is very hard and delicately grained (Bartolucci and Dhakal 1999). The morphology of olive roots depends on the origin of the tree and soil conditions. If plants are raised from seeds, tap roots are formed initially and dominate the entire root system during the first years. On the other hand, if plants are raised from cuttings, adventitious root formation is favoured and they behave as main roots (Fernández et al. 1991). Water and nutrient absorption takes place in the apical region of young roots. The presence of root hairs, small tubular extensions resulting from adaptations of epidermal root cells, enhances the absorption of nutrients and water (Peterson 1989). Olive leaves are persistent, lanceolate, leathery and opposite, limbo of 3-9 cm and a width of 1 to 1.8 cm. The petiole is short, just 0.5 cm. The upper surface is colored dark green and is shiny due to the presence of a thick cuticle and the lower surface is silver-white because it is covered with hairs. Leaves have stomata nestled inpeltate trichomes on their lower

surface (star-like hairs) that restrict water loss due to transpiration and make the olive relatively resistant to drought (Rapoport 1999).

The flowers are white or whitish, the calix is short and 4 –toothed, the corolla is short-tubed with 4 valvate petals, there are stamens, each bearing 10,000 to 15,000 small, light pollen grains. The ovary is 2-loculed, bearing a short style and a capitate stigma. The pistil has two carpels, each containing two ovules but only one is fertilized and thus produces a one-seeded drupe. There are two types of flowers: perfect flowers that contain stamen and pistil, and staminate flowers presenting aborted pistil and functional stamens. The flowers are produced on a cluster ("inflorescence") of about fifteen to thirty flowers, each of which arises in leaf axils of shoots produced in the previous growing season (Bartolucci and Dhakal 1999). The olive fruit is a drupe, which means that it is made up of two main parts: the pericarp and the seed. The pericarp is made up of skin (exocarp), which is smooth, with stomata, flesh (mesocarp), tissue containing oil, and the pit, a lignified shell enclosing the seed (Fabbri et al. 2004).

1.3.2. Olive karyotype and genome size

Many wild and cultivated plants are polyploid, originating in nature basically from unreduced gametes (Schifino-Wittmann 2004), and consequently carry more chromosomic sets. Recent studies show that polyploidization, besides being a dynamic and recurrent process, is accompanied by genetic and epigenetic changes, which has led to wide-ranging genome reorganization at all levels, such as chromosome repatterning, gene silencing, new patterns of gene expression, the action of transposons, intergenomic invasion and concerted evolution (Schifino-Wittmann 2004). Polyploids may be classified according to the origin of their genome into autopolyploids – unique ancestral species – and allopolyploids – original interspecific hybrids (Schifino Wittmann and Dall´Agnol 2003). Most polyploid species are, in contrast to earlier assumptions, of this last type (Schifino-Wittmann 2004).

The olive tree was originated by allopolyploidy, probably from parents whose haploid chromosome numbers were n=11 and n=12 (Breviglieri and Battaglia 1954, cited by Fabbri et al. 2009), giving a commonly diploid (2n=46) species with frequent cross pollination (Alba et al. 2009). However, strong evidence of polyploidy was found, for example, in the north-west African olive subspp. *cerasiformis* (tetraploid) and

maroccana (hexaploid) (Besnard et al. 2008). The coexistence of triploid and diploid individuals was also demonstrated in a population of Laperrine's olive subspecie (Besnard and Baali-Cherif 2009).

Polyploidization can provide the possibility of maintaining higher gene diversity than diploidy (Brochmann et al. 2004, Soltis et al. 2004). Nevertheless, a larger genome size also may bring developmental and reproductive disadvantages, like difficulties in terms of both mitosis and meiosis (Comai 2005). As in other species, variations in olive genome size also occur without modifying ploidy, which can be explained, for example, by the presence of variable content of repeated elements such as tandem repeats or transposable elements (Wendel et al. 2002, Bennetzen et al. 2005). But the causes of genome size variation in olive diploids require further investigation (Besnard et al. 2008).

1.4. Introduction and development of olive growing in Portugal

After the third Punic War, olives occupied a large stretch of the Baetica valley and spread toward the central and Mediterranean coastal areas of the Iberian Peninsula including Portugal (Rugini et al. 2011). The first systematic plantation of olive trees dates from the time of the Roman occupation of the south of Portugal. Some small areas of olive groves are known to have existed in the Alentejo region at this time (Pinheiro 2006). Today the cultivated olives occupy around 640,000 ha in Portugal, mostly in traditional orchards, according to the latest census carried out by the Ministry of Agriculture (MADRP 2004). Fifty-five percent of this area is covered with the Portuguese variety 'Galega vulgar', sometimes referred as 'Galega'.

The Baixo Alentejo (Moura, Serpa and Beja) region has the greatest area of olive orchards, with 64,000 ha, followed by Trás-os-Montes with 52,000 ha and Beira Baixa with 27,000 ha (Fevereiro et al. 2010). The use of different varieties in olive orchards for olive oil production, differentiates the oil produced in each country or region. The distinctive character of each of the six existing commercial Portuguese olive oils is recognised through the system of so-called protected designation of origin (PDO). Five of the six contain cv. Galega vulgar. Other Portuguese varieties which are very well

known and are provide the principal content of olive oil are ‘Cobrançosa’, ‘Verdeal Transmontana’, ‘Cordovil’, ‘Carrasquenha’, ‘Lentrisca’ and ‘Madural’ (Pinheiro 2006).
The degree of use of each variety depends on the region. The expansion of the area of olive groves has become necessary due to requirement for increased exports and the goal of achieving self-sufficiency in terms of consumption (Pinto 2011).

1.5 Olive Propagation

1.5.1. An overview of olive propagation methods

From the origins of its cultivation to the second half of the 19th century, the olive was only propagated agamically, using large cuttings, ovules or rooted suckers (Fabbri et al. 2004). Propagation by seed, especially in allogamous species such as olive, give rise to hybrid plants manifesting the genetic variability of both parents, while vegetative propagation produces clones that are phenotypically and genotypically identical to the mother plant used in the multiplication process (Peixe et al. 2012). Almost as old as propagation by hardwood cutting is the process of propagation by grafting. Dalla Bella (1786), cited by (Peixe et al. 2012), considered this to be the best method of obtaining olive plants with high productivity, vigorous and having longevity. Nowadays, cutting and grafting are still the main processes for olive propagation and seed propagation is therefore confined to the production of seedlings, to be used as rootstocks (Fabbri et al. 2004). Since the 1970s, olive tree production by micropropagation techniques has increased in importance, leading to decades of research in numerous research stations. All propagation techniques have played an important role at one time or another during history, although one technique has usually prevailed at any given time. Choice of propagation method is dependent on available plant material, the production goal and also the technical and financial resources available at the nursery production site.

1.5.2. Plant propagation methods used in thesis trials

1.5.3. Rooting of semi-hardwood cuttings under mist

Mist propagation is a technique employed by plant nurseries to obtain self-rooted plants under specific environmental greenhouse conditions. Semi-hardwood cuttings

are used in this technique and, when compared with traditional hardwood cutting techniques, it considerably increases the amount of vegetal material made available from each mother plant. Besides this, plant uniformity is higher and the process is not dependent on natural environmental conditions for carrying out the technique all year round, when evergreen species are used. These attributes have contributed towards make this the main propagation method used by modern fruit tree nurseries, particularly those aiming at supplying intensive orchards, such as the ones used by the olive oil industry.

To obtain olive semi-hardwood cuttings, the year shoots at the end of the growing season or one-year-old shoots at the beginning of the growing season are collected and prepared in 15-20 cm length portions. After preparation the semi-hardwood olive cuttings have 4-6 nodes, depending on the cultivar, and 4 apical leaves. It is important to collect cuttings from healthy plants, the early morning being the best time for collection because the plant is fully turgid. It is also important to keep cuttings cool and moist until they are placed on the rooting benches. Cuttings present a considerable degree of variation in terms of rooting ability throughout the year; the best time for harvest is spring, but it is possible throughout the whole period of annual growth (Peixe et al. 2012).

Suitable greenhouse conditions such as temperature and humidity are of crucial importance for rooting performance. According to Peixe et al. (2012), the temperature of the rooting substrate should be in the range 24-26 °C, and the greenhouse environment should be maintained at 21-24 °C during the day and about 15 °C at night. Relative humidity inside the greenhouse should be maintained as close to 100% as possible. This is achieved by means of a nebulization system, regulated according to the increase or decrease in environmental temperature: a cooling system. Nebulization is carried out by spraying rooting benches for 5-20 seconds every 10-30 minutes depending on the weather conditions outside the greenhouse, the main goal is to maintain a water film over cutting leaves in order to reduce water loss by transpiration.

Regarding rooting substrate, Perlite is by far the most used type as it maintains the same characteristics over time in all rooting situations. It is a grey-white siliceous

material of volcanic origin, extracted from lava flows (Fabbri et al. 2004). Perlite is placed in rooting beds in layers 10–12 cm thick. Its pH is neutral, and temperature is kept constant at the bottom of cuttings, and there is little temperature exchange with the surface.

With many types of cuttings, the adventitious rooting process depends on the external supply of growth regulators. The auxin indole-3-butyric acid (IBA) is commonly used in olive propagation in semi-hardwood cuttings (Fabbri et al. 2004). The basal part of cuttings (± 3cm) is dipped in a 3,000-5,000 ppm rooting hormone solution for 10-20 seconds and then placed in benches with a substrate.

Fig. 1 shows the main steps in the method of propagation by semi-hardwood that were followed for setting up trials as part of this study.

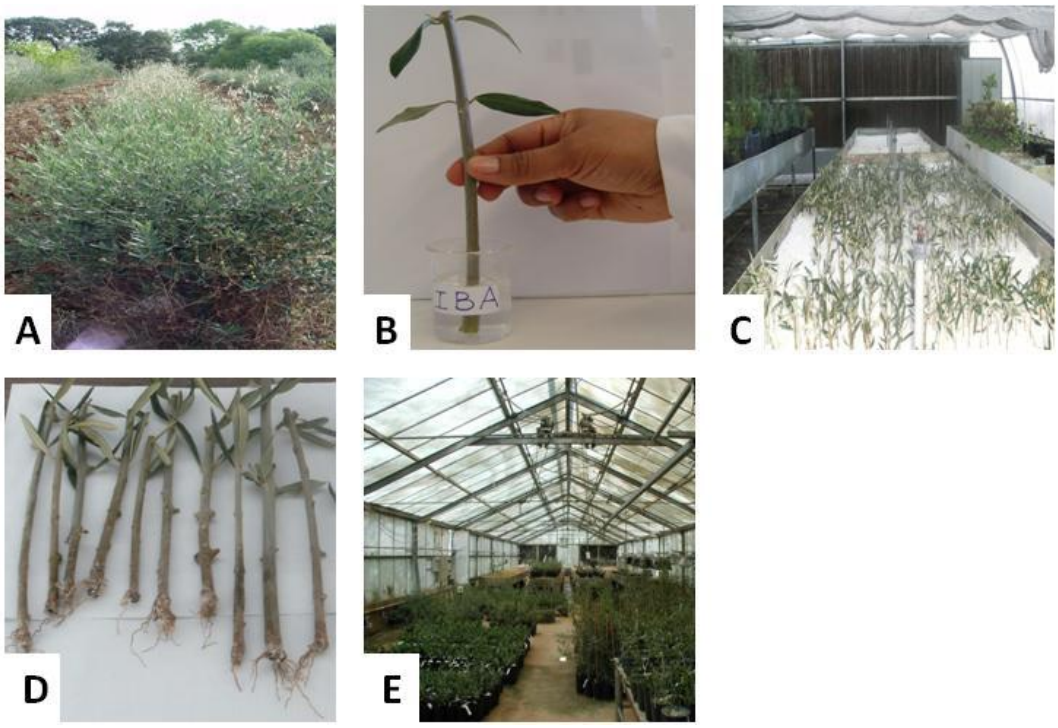

Fig. 1. Procedure followed for the semi-hardwood propagation of *Olea europaea* L. under greenhouse conditions. (A) Plant material used in trial is collected from olive mother field plant and prepared in cuttings 15 cm long, showing 4-6 nodes and 4 apical leaves. (B) The basal part of cuttings (± 3cm) is dipped in IBA**.** (C) Treated cuttings are then placed in benches with a perlite substrate (Font-Macedo 2011). (D) Rooted cuttings (Font-Macedo 2010). (E) Greenhouse acclimatization with plants from greenhouse rooting (Font-Alberto Miranda 2011).

On average, cuttings are kept in rooting benches for around 60 days, although some varieties may need longer (70–80 days). Rooted cuttings (Fig. 1D) are placed in bags (1.5-3L) in a mixture of soil (50-75%) and peat (50-25%) and moved to an acclimatization greenhouse (Fig. 1E) where they stay for 60-120 days, before being taken to the field.

1.5.4 Micropropagation

Micropropagation is a powerful *in vitro* technique which allows for the propagation of pathogen-free genotypes under controlled climatic conditions (temperature, photoperiod, light intensity) and in asepsis (Fabbri et al. 2004).

Sterility is a prerequisite for any type of *in vitro* culture because the culture media are an ideal habitat for the development of fungi and bacteria which colonise plant surfaces. Hence, the media is sterilized and also the initial explant must be disinfected. Culture medium composition is of crucial importance for normal explant development and usually it includes in its formulation macro- and micro-elements, vitamins, aminoacids, sugars growth regulators and water.

The micropropagation of olive varieties was successful in OM medium (Rugini 1984) and subsequently the culture medium was slightly modified by several other researchers who added different growth substances or changed the rooting conditions (Cozza et al. 1997, Mencuccini 2003, Peixe et al. 2007b). The axillary bud technique is applied in the micropropagation of olive trees and can be summarized according to the following procedure (Fig. 2):

1) **Initiation phase:** Leaf-deprived single-node branch portions with intact axillary buds (Fig. 2A) are sown under a laminar airflow hood, in proliferation medium contained in culture flasks. These are then placed in a thermostat-controlled growth chamber at 24 °C/21 °C (±1 °C) day/night temperature with a light photoperiodof 15 h, under cool white fluorescent light (36 µmol $m^{-2}s^{-1}$) for 30 days (Fig. 2B).

2) **Proliferation phase:** The microshoots obtained in the first phase are separated from their basal generated calli and subcultured for development under the

same previous culture conditions for 45-50 days (Fig. 2C). Then, the upper halves are partially de-leafed (in accordance with Fig. 2D1) for rooting culture (Fig. 2E), whereas mono-nodal segments are prepared from the bottom halves of the microshoots developed (Fig. 2D2), to be employed in a new multiplication cycle (Fig. 2F).

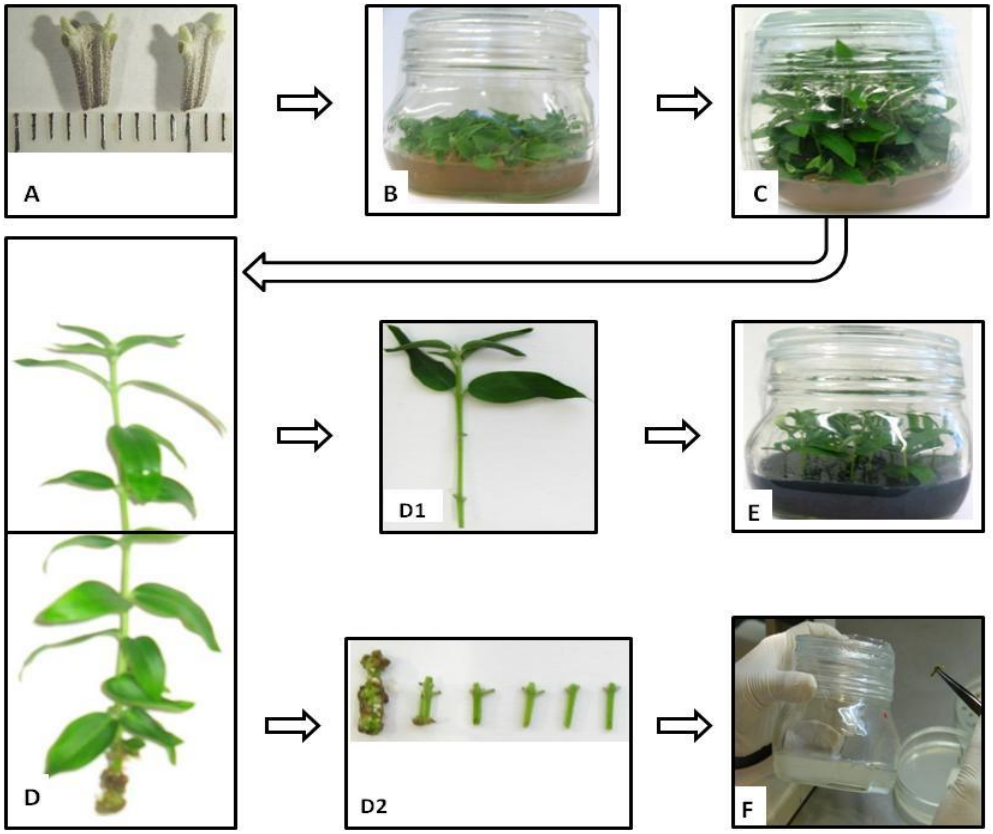

Fig. 2. Olive microshoot multiplication by means of the axillary bud technique. (A) Mono-nodal explants prepared for *in vitro* sowing. (B) 30 days culture in proliferation medium – initiation phase. (C) Microshoot development for 45-50 days subculture in proliferation medium – proliferation phase. (D) Developed microshoot. (D1) Upper half from developed microshoot, prepared for rooting culture (E). (D2) Mono-nodal segments from bottom half of developed microshoots, prepared for a new multiplication cycle – restarting initiation phase (F).

3 - **Rooting phase:** The emergence of roots is promoted by a growth regulator (for example IBA), which can be applied at the base (~0,5cm) of microshoots (Fig 2.D1) for 10 seconds. After this, explants are inoculated *in vitro* in semi-solid OM culture media (Fig 2.E) (Rugini 1984), devoid of growth regulators. The containers with microshoots

are placed in a thermostat-controlled growth room, at 24 ºC / 21 ºC (±1 ºC) day/night temperature and a light period of 15 h, under cool white fluorescent light (36 µmol $m^{-2}s^{-1}$).

Rooted explants (Fig. 3A) are ready in about 3–6 weeks and transplanted into containers with substrates such as perlite and peat, and placed in the greenhouse for acclimatization. Following acclimatization, plants with well-developed roots (Fig. 3B), are removed from containers and transplanted to a permanent site.

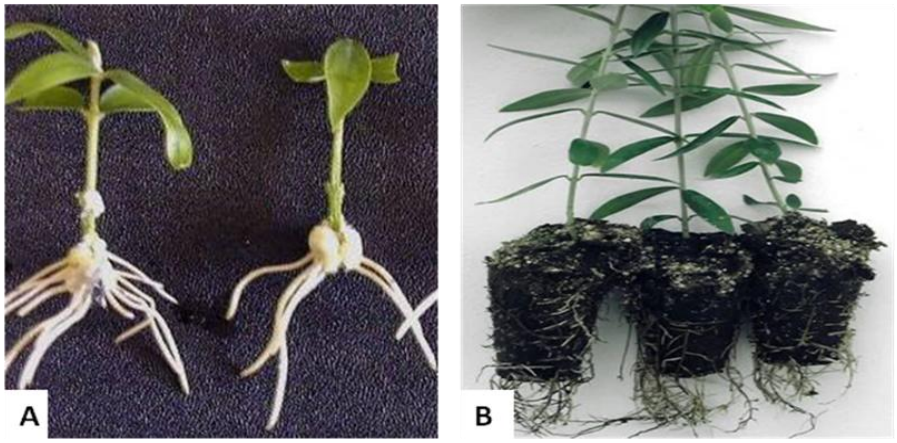

Fig. 3. (A) Rooted explants. (B) Plants acclimated, ready to be transplanted to a permanent site (Font- Peixe et al. 2012).

1.6. Adventitious root formation

1.6.1. Importance and general characterization

Adventitious root formation (ARF) is a cell dedifferentiation process which occurs in different organs in response to a combination of different endogenous and exogenous signals (Pop et al. 2011). ARF has many practical implications in horticulture and agronomy, involving several commercial applications, because many plant species are difficult to root (Davies et al. 1994, Kovar and Kuchenbuch 1994).

The plant organs used for asexual reproduction in vegetative propagation are: stems, roots and leaves. ARF can be divided into different phases, each with different requirements. Three different phases are recognized: i) induction, which comprises the

initial molecular and biochemical modifications; ii) initiation, characterized by cell divisions and root primordia establishment and organization; iii) expression, in which growth and emergence of adventitious roots occur (Li et al. 2009).

Fabbri et al. (2004) briefly described ARF in semi-hardwood olive cuttings. The first events are related with plant response to wounding and aimed at isolating explants from the environment, in order to avoid water loss from the cut area: vessels, which can cause abundant water loss, are plugged with gums (tyloses). Living cells from several tissues below the cut surface abandon their cytological and physiological commitment (dedifferentiation), regain the ability to divide and start division, thus differentiating into specialized parenchyma cells (thereby producing callus). Finally, new roots emerge from concrete differentiated cells. According to Hartmann et al. (1997), callus formation preceding rooting is normal when indirect root formation occurs (Fig 4A), being a common feature in hard-to-root explants, while other varieties do not present calli (Fig 4B).

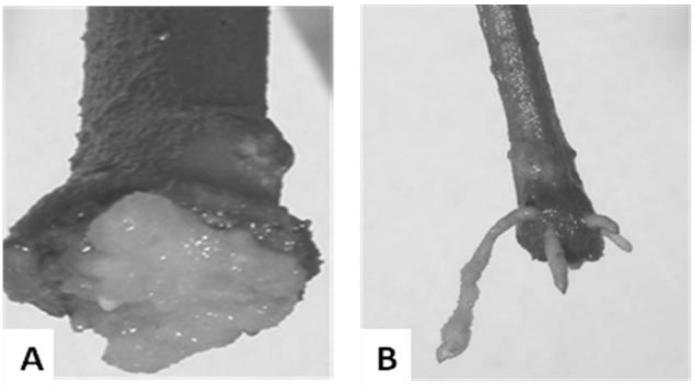

Fig. 4. Olive cuttings: developing callus (A) and developing roots (B). Photos adapted from Fabbri et al. (2004).

1.6.2. Factors affecting ARF efficiency

ARF is a quantitative genetic trait regulated by both environmental and endogenous factors (Pop et al. 2011), whose interaction is still poorly understood (Davis et al. 1988). In the following chapters, some relevant aspects of factors which can affect root formation at the stem cuttings bases are briefly presented.

1.6.2.1. The Genotype

Cutting characteristics have been associated with ARF ability (Hartmann et al. 1997), but the result of this association varies according to the species (Harrison-Murray and Knight 1997). Genotypic differences in rooting have been previously documented for a number of woody species, *Persoonia virgata* (Bauer 1999), *Acacia baileyana* (Schwarz et al. 1999) and *Eucalyptus resinifera* (McComb and Wroth 1986).

Among olive varieties, the capacity for adventitious root formation has proved to be extremely variable (El-Said et al. 1990, Fouad et al. 1990, Salama et al. 1987). All Mediterranean countries have one or two economically important olive varieties showing recalcitrance. This is the case for the cvs. 'Gordal sevilhana' from Spain, 'Kalamata' from Greece, 'Domat' from Turkey (Gerakakis and Özkaya 2005), 'Gentile di Larino' from Italy (Sebastiani et al. 2004), and 'Galega vulgar' from Portugal (Peixe et al. 2007a).

1.6.2.2. Developmental stage of stock plant

Adventitious root formation in woody plants is also intrinsically linked to the developmental stages of the plant. It is well known that the majority of mature woody plants do not root easily (Sedira 2006). This includes cuttings from most adult apple trees which are extremely difficult, if not impossible, to root (Brown 1975). Many cells and tissues cannot acquire competence to regenerate new tissues and organs even under favourable conditions (Sedira 2006). A study carried out with mature Griselina showed that the rooting stimulus was less effective in mature plants and that the target cells were less responsive than those in juvenile plants (White and Lovell 1984). Hence, it may be concluded that a high potential for adventitious rooting is considered a juvenile characteristic. Before taking cuttings, it is also worth considering the developmental stage of the plant. For example, during flowering and fruiting there is a diversion of metabolites involved in such processes, to the detriment of metabolites which are responsible for rooting (Oliveira et al. 2002).

Regarding the position of cutting from the branch producing the cutting, in the olive, apical, medial and basal cuttings were shown to have rooting response; the first two

showed better results (Abousalin et al. 1993). However, Al-Imam (2011) reported that olive semi-hardwood basal cuttings showed higher rooting percentage when they were treated with an optimal concentration of IBA. Both apical and basal stem cuttings of fever tea (*Lippia Javanica* L.) were able to root when the ideal propagation medium for pine bark was chosen (Soundy et al. 2008).

Taking into account all these scientific data it can be concluded that the best selection for cutting for rooting is dependent on multifactorial interaction.

1.6.2.3. Seasonality for collecting cuttings

Cuttings of some species, like quince and poplar, root well when samples are harvested at any time of the year, while others, like cherry and olive, root successfully only during certain periods of the year (Hartmann and Brooks 1958, Hartmann and Loreti 1965). Cuttings of many woody plants taken early in the growing season rooted easily in contrast with those taken later (Blakesley et al. 1991).

Olive cuttings showed a similar marked seasonal variation in rooting ability, achieving the highest level (80%) in spring–summer (May-June) and the lowest level (20-30%) in winter (Mancuso 1998).

The presence of auxin and nutrients are required for rooting and there are two periods of the year during which levels of these substances are favorable. The first is in late spring or early summer, when vegetative shoot growth is at its height and cuttings have a higher content of these substances. The second is in the autumn, before the drop in environmental temperature leads to a decrease in plant physiological activity. In this period, cuttings have maximum levels of carbohydrates necessary for respiration, which is also important for the development and growth of roots (Council 2008).

1.6.2.4. Physiological stage of mother plant

Cutting ARF depends on the internal conditions of the mother plant and the environmental conditions in which it is placed (Bueno 1995). The availability of water and a range of nutrients, such as nitrogen, phosphorus, zinc, potassium, calcium and

boron, are involved in diverse metabolic processes associated with dedifferentiation and root formation (Blazich 1988). Root development is also strongly influenced by the nutritional status of the mother plant from which the cutting was taken (Hartmann and Kester 1983). Water content in cutting tissue is important and depends on plant transpiration rates. High humidity (Fachinello 1986) and low temperature (Hartmann and Kester 1983) prevents tissue from drying, thus favoring further cutting rooting.

1.6.2.5. Growth regulators

Different types of plant growth regulators have been demonstrated to influence adventitious rooting (AR) (Fabbri et al. 2004). Frequently, plant growth regulators are conjugated (i.e. covalently bound) with other molecules and therefore they are physiologically inactive. Conjugation reactions are mostly reversible and thus constitute a very flexible mechanism for the control of endogenous growth regulator levels by the plant (Machakova et al. 2008).

In vegetative propagation, plant growth regulators are used in the root induction phase to increase the number and quality of further developed roots. In olive the exogenous application of auxins is common, their endogenous concentrations constituting a limiting factor for rooting (Del Rio et al. 1986, cited by Oliveira et al. 2003).

In general, it is accepted that there are five major classes of growth regulator: Auxins, Cytokinins, Gibberellins, Ethylene and Abscisic acid.

Several reports have indicated the involvement of exogenous and endogenous auxins in the initiation of adventitious rooting (Fabbri et al. 2004, Ludwig-Müller 2009, Asl moshtaghi and Shahsavar 2010). As early the 1930s, two groups of researchers (F. Kölg and AJ Haagen-Smith in Holland, and K. V. Thimann in the United States) identified the indole-3-acetic acid (IAA) as auxin. Subsequently, other natural auxins were discovered (phenyl-acetic acid and 4-Cl-indole-3-acetic Acid), but IAA is the most abundant auxin reported so far (Lacerda et al. 2007). IAA is synthesized from Tryptophan through the shikimate pathway or by Tryptophan independent routes (Zhao 2010). Plants can also obtain IAA by β-oxidation of indole-3-butyric acid (IBA), a second endogenous auxin, or by hydrolysing IAA conjugates, in which IAA is linked to amino acids, sugars or peptides

(Woodward and Bartel 2005). Auxins conjugated with other molecules do not present physiological activity. For example, IAA conjugates may be glycosyl esters or amide - linked to various amino acids and peptides. IAA conjugation helps to maintain the homeostasis, both by inactivation and by providing a reservoir of IAA, since it can be released by hydrolysis (Staswick et al. 2005). IAA degradation occurs mainly by oxidation in two ways: with or without decarboxylation (Machakova et al. 2008).

IBA is the most widely used auxin, even for root initiation in conventional cuttings, since it is naturally found in plants, it is highly stable to light, possesses low toxicity and has local action (Fabbri et al. 2004, Hartman et al. 1997, Pio et al. 2005). Probably this molecule has a direct biological effect (Van der Krieken et al. 1992), but it has been shown that it is readily converted to IAA. This conversion has been measured *in vivo* in several plant species, such as apple (*Malus pumila*) (Alvarez et al. 1989), Scots pine (*Pinus sylvestris*) (Dunberg et al. 1981), European aspen (*Populus tremula*) Merckelbach et al. 1991), pear (*Pyrus communis*)(Baraldi et al. 1993), Grape vine (*Vitis vinifera*) and olive tree (*Olea europaea*) (Epstein and Lavee 1984).

Peixe et al. (2007a) reported the application of IBA 17mM for 20 s in semi-hardwood cuttings of two cultivars: 'Galega vulgar' – difficult to root (10%) and 'Cobrançosa' – easy to root (70%).

Varietal differences in AR capacity have been attributed to distinct auxin metabolism (Epstein and Ludwig-Müller 1993, Blazkova et al. 1997, Alvarez et al. 1989). The presence and/or accumulation of free auxins in the base of the cuttings normally increase prior to ARF (Ayoub and Qrunfleh 2006, Pio et al. 2005). The auxins produced in the leaves or buds moves naturally to the bottom of the cutting and accumulates with sugars and other nutrients (Janick 1996), helping the cutting to root (Nanda et al. 1971) (see 3.2.9 carbohydrates).

The applied concentration of auxins should also be considered. According to Frasseto 2007, the use of high concentrations of auxins may kill the cells in the cutting base, causing excessive cell proliferation and callogenesis or inhibit root growth.

The second class of growth regulator is cytokinins, a group of chemicals that influence cell division and shoot formation. They also help delay senescence or the aging of

tissues, and are responsible for mediating auxin transport throughout the plant, and affect internodal length and leaf growth (Sipes and Einset 1983)

Cytokinins often have an inhibitory effect on adventitious rooting, acting as auxin antagonists. Hence, a balance between stimulatory and inhibitory effects on rooting initiation is essential (Hartmann and Kester 1983, Wendling et al. 2000, Ayoub and Qrunfleh 2006). A high concentration of auxin/cytokinin ratio favors rooting (Hartmann et al. 1997, Salisbury and Ross 1992). In apple, for microcuttings it was found that suitable hormonal conditions for rooting, i.e. a high level of auxins and a low levels of cytokinins, are required during the induction phase (Ludwig-Müller 2009). However, early root initiation in decapitated in pea cuttings was promoted after the application of low concentrations of cytokinins (Eriksen 1974).

Another class of growth regulator is the so-called gibberellin group (GAs), a class of tetracyclic diterpenoid hormones that regulate many developmental processes such as seed germination, root and shoot elongation, flowering and fruit patterning (Fleet and Sun 2005, Yamaguchi 2008). In contrast to auxins, and like cytokinins, gibberellins have a negative role in ARF (Hartmann and Kester 1983, Davis et al. 1988). According to Arteca (1995), gibberellins stimulate vegetative growth, which competes with root formation, thereby blocking the action of auxins after root induction. However, Ford et al. (2002) showed that GA3 pre-treatment of *P. avium* stock plants causes an increase in shoot growth rate and also improves the rooting of cuttings subsequently taken from the treated plants.

Ethylene (C_2H_4) gas, which is synthesized from methionine in many tissues in response to stress (Pech et al. 2004), such as disease, radiation or mechanical wounding (Walley et al. 2007), and is the fruit-ripening hormone, is often considered as another type of plant growth regulator (Pech et al. 2004).

Ethylene affects root initiation and development in some plants. In olive cv. ´Maurino`, basal treatment of cuttings with 10 mM 1-aminocyclopropane-1-carboxylic acid (ACC), the direct biosynthetic precursor of ethylene, in combination with IBA (4,000 ppm), showed a positive effect on rooting only when cuttings were treated for 1 to 3 hours after preparation (Bartolini et al. 1986). It has been postulated that auxin works by

enhancing ethylene biosynthesis. However, the role of ethylene in the rooting process is not completely understood (Fabbri et al. 2004).

Abscisic acid (ABA) is the last of the main classes of plant growth regulators. It coordinates responses to stressors such as drought, extreme temperature and high salinity, as well as regulating non-stress responses including seed maturation and bud dormancy (Sheard and Zheng 2009). It also lays an important role in stomatal closure control, decreasing transpiration and preventing water loss (Zhang et al. 1987).

ABA is important for rooting, since it antagonizes the effects of gibberellins and cytokinins (Hartmann et al. 1997). According Steffens et al. (2006), root emergence and elongation are distinct phases of adventitious root growth that are regulated through different networking between ethylene, GA and ABA signaling pathways, and different interactions between the same hormones may be a means to achieving root emergence and adjusting the growth rate of emerged adventitious roots.

1.6.2.6. Enzymes

Oxidative enzymes

The roles and the importance of peroxidases (POX) and polyphenol oxidases (PPO) during rooting were reported by several authors (Moncousin and Gaspar 1983, Gaspar et al. 1992, Berthon et al. 1989, Rival et al. 1997, Rout et al. 1999, Cheniany et al. 2010, Fu et al. 2011).

Classical plant POX are heme-containing enzymes that catalyze the oxidation of a diverse group of organic compounds. Among POX isozymes, cationic isoforms were reported as being able to catabolize IAA (Gazaryan et al. 1999), thus influencing rooting. The effect of POX inhibition on *Arabidopsis thaliana* root and root length was greatly reduced (Dunand et al. 2007). POX activity sharply decreases during rooting induction, increases during initiation, and gradually decreases during the expression phase (Hatzilazarou et al. 2006, Metaxas et al. 2004, Rout et al. 2000, Syros et al. 2004). Due to these close relationships, POX has been proposed as a biochemical marker of the successive rooting phases (Metaxas et al. 2004, Rout et al. 2000, Syros et al. 2004).

PPO are copper-containing enzymes localized in the thylakoids of plastids. These enzymes catalyse the oxidation of phenolic compounds into quinines (Vaughn et al. 1988). They are also involved in the defense mechanisms of plants against different environmental stresses (Thipyapong et al. 1995). Furthermore, PPO play a key role in rooting (Gonzalez et al. 1991) and are involved in regulating the synthesis of phenolic precursors needed for lignin biosynthesis during root differentiation (Haissig 1986).

1.6.2.7. Hydrogen Peroxide (H_2O_2)

It is well known that plant ARF can be stimulated by the exogenous application of hydrogen peroxide (H_2O_2), but the mechanism for this physiological response is still unclear (Sebastiani and Tognetti 2004). Li et al. (2009) demonstrated that H_2O_2 works as a signaling molecule in AR of cucumber.

In olive cuttings, the early rooting rate was improved with the exogenous application of H_2O_2, together with IBA and the polyamine putrescine (Ozkaya et al. 1993, Rugini et al. 1990, 1997). Moreover, in nursery propagation the application of H_2O_2 with IBA significantly improved rooting in olive varieties with distinct AR abilities: 'Frantoio' and 'Gentile di Larino', with high and low rooting efficiencies, respectively (Sebastiani et al. 2004).

1.6.2.8. Carbohydrates

Carbohydrates, which are produced through photosynthesis and stored as starch grains, are particularly important as an energy source in cutting rooting process (Fabbri et al. 2004). Besides this, the biosynthesis of these molecules provides structural carbon for AR (Veierskov 1988). Internal carbohydrates might also interact with endogenous growth regulators and have a stimulatory effect on root initiation (Wiesman and Lavee 1995). The association of these carbohydrates with auxin stimulation contributed to ARF (Hartmann and Kester 1983). Several studies show the requirement for a certain balance between auxins and carbohydrates for optimal root development, since there is a continuous loss of soluble starch and sugars in the base of the cutting (Davis 1983, Del Rio and Caballero 1991, Ruiz and Loreto 1998).

Carbohydrates might also affect auxin metabolism (Wiesman and Lavee 1995), thus influencing AR.

Carbohydrates, particularly mannitol (the most abundant sugar alcohol in cuttings), were responsible for the high rooting ability of the olive cv. Arbequina, since a high concentration at the beginning of AR was observed (Denaxa et al. 2012).

1.6.2.9. Secondary metabolic compounds

Phenolic compounds constitute a large and diverse group of molecules which includes many different families of plant aromatic secondary metabolites. These phenolic derivatives are the most abundant secondary metabolite in plants and can be classified into non-soluble compounds, such as condensed tannins, lignin or cell-wall bound hydroxy cinammic acids and soluble compounds, such as phenolic acids, phenylpropanoids, flavonoids and quinones. All these groups are involved in many metabolic processes in plants and animals (Rispail et al. 2005). Phenolic compounds are considered as playing a key role in AR induction, acting in synergy with auxins (James and Thurbon 1981, Rout 2006). Rout (2006) reported that phenolic compounds can be used as rooting enhancers in the tea plant. Phenolic acids, flavonoids and lignin are involved in plant defense under oxidative stress and are formed through the phenylpropanoid route, which is very important in secondary metabolism (Iriti and Faoro 2009). The involvement of lignin and phenolic acids in adaptive plant growth regulation, differentiation and organogenesis has been explained by several research groups (Fu et al. 2011, Ozyigit et al. 2007, Sircar et al. 2012). Phenylpropanoid derivatives and lignin are also involved in the regulation of cell-division and differentiation (Cvikrova et al. 2003, Palama et al. 2010, Tamagnone et al. 1998).

Phenylpropanoid derivatives were also related with rooting. Fu et al. (2011) reported that their accumulation stimulates *in vitro* AR of the peony tree (*Paeonia suffruticosa* Andr.) plantlets. *In vitro*, phenolic acids modulate POX activity (involved in auxin degradation): monophenols activate this enzyme, whereas di- and polyphenols inhibit it (Lee et al. 1982). Phenolic compounds probably act as antioxidants, protecting IAA

from oxidation by changing its oxidative metabolism of IAA and thus regulating the amount of auxin of the tissues (Fogaça 2003).

Flavonoids are biochemical markers of rooting. Rooting capacity *in vitro* depended on the levels of endogenous flavonoids in a clone of *Eucalyptus gunnii*. These molecules could have some direct influence on the rooting processes of *in vitro* explants (Curir et al. 1990). In contrast, lignin accumulation led to cortex suberization, responsible for bad rooting ability in 16 woody plant species (Williams et al. 1984).

1.7. Anatomic aspects of the origin of adventitious roots

Adventitious root primordia can arise from cells with different tissue origin. For different plant species, there are references to the origin of adventitious rooting in the epidermis, cortex, cambium, secondary phloem, pericycle and vascular bundles (Ono and Rodrigues 1996). When xylem and secondary phloem are present, adventitious roots can be originated from the young tissue of secondary phloem, or can even derive from: phloem parenchymatic cells between vascular bundles; vascular ray; cambium; or the calli developed in the base of shoot cuttings (Hartmann et al. 2002, Lovell and White 1986, Messina and Testolini 1984). Peixe et al. (2007a) reported a differential origin of morphogenic field for adventitious roots in semi-hardwood cuttings of two olive varieties with a different behavior for AR development. 'Cobrançosa', an easy-to-root cultivar, developed new roots from vascular cambium or adjacent tissues, whereas 'Galega vulgar', known as being recalcitrant for AR, presented the morphogenic field originated from the parenchymal cells of the calli developed on the cutting base. This distinct potential for developing adventitious roots is mainly related to genotype rather than to anatomical differences. Both varieties present a sclerenchymatous ring. This tissue is characterized by the suberization of the cortex and the deposition of lignin and cellulose in the cell wall, representing an anatomical barrier for root primordial development, which was reported in different plant species (Dalet and Cornu 1988, Williams et al. 1984). This is described in greater detail in Chapter 4.

1.8. Alternative oxidase (AOX): functional marker (FM) development for adventitious rooting in Olive

1.8.1. AOX characterization

Mitochondria of plants have two important electron transport chains (ETCs): electrons can flow through the cytochrome c pathway that generates the proton-motive force used for ATP (adenosine triphosphate) synthesis, or through the alternative respiratory pathway (Fig 5), that channels the electrons from ubiquinone to oxygen through AOX and is not coupled with ATP production (Li et al. 2012). The alternative oxidase are widely distributed by Kingdoms (plant, fungi, algae and protozoans, arthropods) except Arachaebacteria (McDonald and Vanlerberghe 2004, McDonald and Vanlerberghe 2008).

The AOX is an integral monotopic membrane protein that is tightly bound to the inner mitochondrial membrane from matrix side (Berthold and Stenmark 2003) and is a 32-37KD (Vanlerberghe and McIntosh 1997). This enzyme catalyses the oxidation of quinol and the reduction of oxygen to water (MacDonald 2008). This enzyme can be in the form non-covalently bound (reduced) dimer or a covalently bound (oxidized) dimer (Umbach and Siedow 1993). Activation of AOX occurs when the enzyme is in the reduced form and requires the presence of specific α-keto acids, an example is pyruvate (Millar et al. 1993, Millennaar et al. 2002).

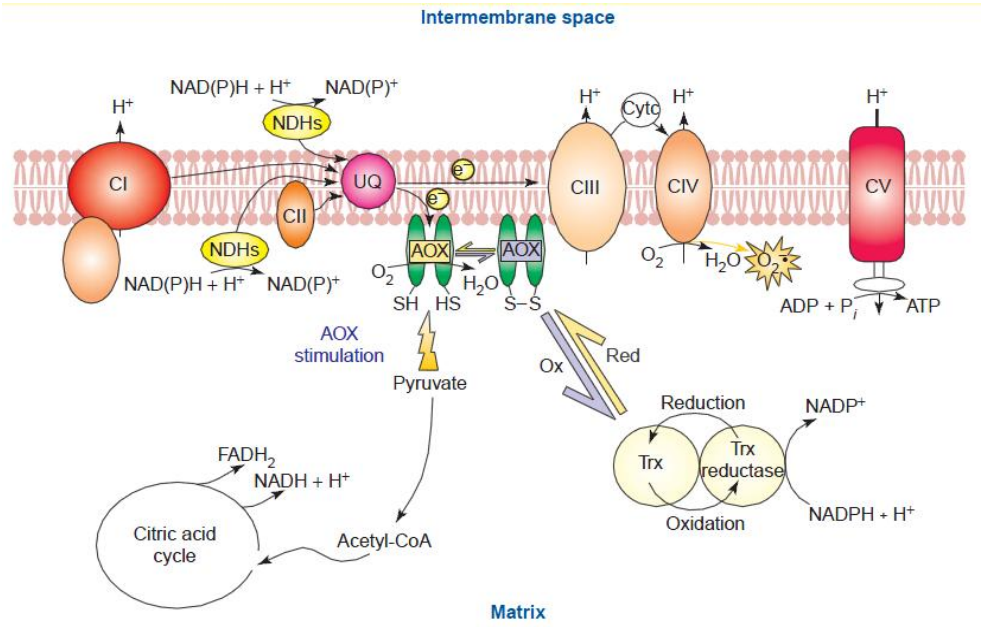

Fig.5. Organisation of electron transport chain components in the inner membrane of plant mitochondria. Abbreviations: AOX, alternative oxidase; CI, NADH: ubiquinone oxidoreductase; CII, succinate: ubiquinone oxidoreductase; CIII, Cytochrome bc1 complex; CIV, cytochrome c oxidase; CV, ATP synthase; Cytc, cytochrome c; NDHs, rotenone-insensitive NAD(P)H dehydrogenases; Pi, inorganic phosphorus; Trx, thioredoxin; UQ, ubiquinone pool (Font-Arnholdt-Schmitt et al 2006).

AOX can be inhibited by hydroxamic acids depending on concentrations. Evidently the functional group from hidroxamic acid, -CONHOH is required for the inhibitory effect (Schonbaum et al. 1971). The pathway's ability to reduce oxygen to water requires a transition metal (such as iron) and SHAM acts as an Fe (III) chelator (Sprienger et al. 1987). AOX proteins has been identified as diiron carboxylate proteins (Berthold and Stenmark 2003). The active sites of these proteins contain a four-helix bundle—a common structural element for this group - that acts as a scaffold to bind the binuclear diiron center. In general, the two iron atoms are ligated by four highly conserved glutamate/aspartate residues and two histidine residues, an arrangement which results in yields a characteristic signature motif (Siedow and Umbach 1995, Andersson and Nordulund 1999, Berthold et al. 2002). The di-iron site positions confer resistance

to the AOX inhibitor salicylhydroxamic acid (SHAM) (Berthold 1998). According some author, the helix 1 (hydrophobic region) of the AOX is related with the inner membrane forming the ubiquinol binding site (Andersson and Norlund 1999, for review see Albury et al. 2009).

An important characteristic of AOX has been revealed in non-thermogenic plants that are two highly conserved cysteine residue placed towards the N-terminal hydrophilic domain of AOX, theses residues are involved in reduction/oxidation regulation and in α-keto acid activation (Rhoads et al. 1998).

The importance of this cysteine has been reported when Cys is replaced by another amino acid, that can influence the behavior of AOX, for example in Arabdopsis. When Cys1 is replaced by Ala, the AOX protein is unable to be oxidised by diamide (Rhoads et al. 1998, Vanlerberghe et al. 1998) and when Cys 1 is replaced by Ser, the AOX is activated by succinate but not by pyruvate (Djajanegara et al. 1999, Holtzapffel et al. 2003). Cys2 may also be involved in regulating AOX activity through interaction with the α-keto acid glyoxlate (Umbach et al. 2002).

One demonstrated function of the alternative pathway of respiration in thermogenic plants (such as arums as dead horse arum and sacred lotus) is the heat production during anthesis, then volatilizing aromatic compounds to attract pollinators (Meeuse and Buggeln 1975, Angioy et al. 2004, Watling et al. 2006). However, the function of the alternative pathway in non-thermogenic tissues is still unclear (Florez-Sarasa 2011). All organisms produce a range of reactive oxygen species (ROS) during the course of normal metabolic processes. ROS, if not effectively and rapidly removed from cells, can damage a wide range of macromolecules, likely leading to cell death (Maxwell et al. 1999). Mitochondrial ROS might have important signaling functions within cell, but their excessive generation may cause oxidative damage to the mitochondrion. By preventing the over-reduction of the respiratory chain, AOX could act to reduce ROS generation (Apel and Hirt 2004). An inverse relationship exists between the levels of AOX and ROS in the mitochondrion. It was observed that AOX induction was in a central position to diminish ROS production, thus functioning to enhance the plant basal defenses and collaborating to maintain the homeostasis. For

example, AOX seems to help the plants to cope with systemic tobacco mosaic virus (TMV) infection (Liao et al. 2012).

The role of AOX was indicated also in diverse other types of stress, like hypoxia. Under such a stress, nitric oxide (NO) is produced in roots of *Arabdopsis thaliana* wild-type. Aconitase (enzyme found in mitochondria and involved in the tricarboxylic acid citric (TCA) cycle that mediates the conversion of citrate to isocitrate) is inhibited by NO, and consequently the level of citrate increases, which thereafter induces AOX. This pathway leads to a shift of plant metabolism towards aminoacid biosynthesis and provides an important adaptation strategy of plants to low oxygen conditions (Gupta et al. 2012).

Other abiotic stresses have been related to increasing AOX protein levels such as: high light (Noguchi et al. 2005), low light (Florez-Sarasa et al. 2009), low temperatures (González-Meler et al. 1999, Watanabe et al. 2008). AOX has been expressed in many tissues and growth stages indicating that its role is not only related with oxidative stress (Clifton et al. 2006, Ho et al 2007). A role of AOX was reported for the development of fruits (Sluse and Jarmuszkiewicz 2000, Considine et al. 2001), leaves (Florez-Sarasa et al. 2007) and roots (Millar et al. 1998).

Much research has been performed on molecular aspects of different components of the plant mitochondrial electron transport chain during last decade (Millar et al. 2008) and great importance has been given to AOX proteins (Albury et al. 2009, McDonald et al. 2009, Polidoros et al. 2009, Vanlerberghe et al. 2009) and genes (Cardoso et al. 2009, Ferreira et al. 2009, Campos et al. 2009, Frederico et al. 2009, Polidoros et al. 2009).

AOX isozymes are encoded by a small nuclear family consisting of two subfamilies (1 and 2). The predominant structure of genomic *AOX* sequences consists of four exons interrupted by three introns at well conserved positions. Nevertheless, evolutionary intron loss and gain has resulted in the variation of intron and exon numbers. Thus, the number of introns varies from two to four and that of exons from three to five (Polidoros et al. 2009). Typically, although not exclusively, *AOX1* genes are induced by many different kinds of stress, whereas *AOX2* genes are expressed in a constitutive or

developmentally regulated way (Polidoros et al. 2009). However, *AOX2* genes also appear to play a role in plant stress responses (Clifton et al. 2005, Costa et al. 2007).

In monocots only genes belonging to the *AOX1* subfamily have been identified while in dicots genes from both subfamilies are present (Whelan et al. 1995, 1996, Saisho et al. 1997, Considine et al. 2001, Karpova et al. 2002, Takumi et al. 2002, Borecky et al. 2006, Clifton et al. 2006, Li et al. 2008). In dicots have been identified three to four *AOX1* and two *AOX2* genes. Saisho et al. (1997) reported the existence in *A. thaliana* of four *AOX1* genes (*AtAOX1a*, *AtAOX1b*, *AtAOX1c* and *AtAOX1d*) and one *AOX2* gene (*AtAOX2*). However, there are several examples showing the existence of a single gene belonging to the *AOX1*-subfamily and two genes belonging to the *AOX2*-subfamily, as it is the case of soybean (*Glycine max*) (Thirkettle-Watts et al. 2003) and cowpea (*Vigna unguiculata*) (Costa el al. 2010). Campos et al. (2009) isolated *Daucus carota* L. *AOX* genes, reporting two genes in each *AOX* subfamily (*DcAOX1a, DcAOX1b* and *DcAOX2a, DcAOX2b*). This pattern was different from those known up to then, showing that the number of *AOX* genes can be variable among the species.

1.9. Functional marker approach

A FM is a DNA (deoxyribonucleic acid) marker with defined functionality of its sequences (Andersen and Lübberstedt 2003), being a DNA marker a small DNA region that shows sequence polymorphism among individuals within a species (Botstein et al. 1980, Gupta et al. 2002). FM development requires the existence of a gene directly related with a specific trait, whose alleles show polymorphic sites associated to phenotypic differences (Gupta et al. 2002). DNA polymorphisms such as single-nucleotide polymorphisms (SNPs) or insertion/deletions (InDels) may directly contribute to a phenotype (Thornsberry et al. 2001). SNPs are less mutable than other markers, particularly microsatellites. Their low rates of recurrent mutation make them evolutionarily stable. They may be excellent markers for studying complex genetic traits and for the understanding of the genomic evolution (Jehan and Lakhanpaul 2006). Therefore, SNPs, so as InDels, are reasonable starting points for FM development (Juwattanasomran et al. 2012, Cardoso and Arnholdt-Schmitt 2013). FMs are superior to random DNA markers, such as amplified fragment length

polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs), for genetic trait characterization due to their complete and direct linkage to trait locus alleles (Andersen and Lübberstedt 2003).

FMs allow application of markers in populations without prior mapping, the use of markers in mapped populations without risk of information loss due to recombination and better representation of genetic variation in natural or breeding populations. Among the advantages of FMs are : (i) more efficient fixation of alleles in populations; (ii) controlled balancing selection; (iii) screening for alleles in natural as well as breeding populations; (iv) combination of FM alleles affecting identical or different traits in plant breeding; and (v) construction of linked FM haplotypes. A of limitation for FM development is that only 10% of all genes have been functionally characterized for model species, whereas (for non-model species) this number is probably less than 5%. However, genes can be characterized in a biological sense, but this might not be agronomic importance (Andersen and Lübberstedt 2003).

Candidate gene sequences related with specific phenotypes have been reported, such as: for height of plants in cereal the gene called Dwarf8 orthologs (Ikeda et al. 2001), for fruit size in tomato (*Lycopersicon ssp*.) the gene fw2.2(Nesbitt et al. 2002), for Food quality in rice (*Oryza sativa*) the gene called GBSS (Ayres et al. 1997),for general stress response in several species the gene called ERF transcription factors (Singh et al.2002), for flowering time in maize (*Zea mays*) the gene Dwarf8 Maize (Thornsberry et al. 2001), for adaptation to several abiotic stresses such as drought and salt in *Phaseolus vulgaris* L. Recently the *pyrroline-5-carboxylatesynthetase* (*P5CS*) *P5CS* gene has been used for FM development (Chen et al. 2010) .

Finally, the potential importance of SNPs in AOX genes has been indicated by Abe et al (2002) by verifying that an *AOX* SNP in rice (*Oryza sativa* L.) conferred low temperature tolerance. An amino acid substitution was induced by mutagenesis in AOX1a, which corresponded to isoforms in the protein that were tightly linked to a quantitative trait loci (QTL) for low temperature of anthers (Abe et al. 2002). Tolerant AOX protein presented a band correspondent to a mass of 32-kDa, while the no tolerant protein showed a 34-kDa band. This difference was due to the substitution of Lys (71) by Asn,

caused by a SNP between alleles of *OsAOX1a*. This research in addition to the findings of Lee Hansen's group (2002), who showed the importance of AOX for acclimation, stimulated the present research approach of the thesis (Arnholdt-Schmitt 2005a, Arnholdt-Schmitt et al. 2006, Arnholdt-Schmitt B 2009, Hansen et al. 2009, Polidoros et al. 2009).

1.10. AOX potential for Functional Marker development

As previously seen one of the prerequisites for the development of a functional marker is the presence of interesting polymorphisms. Several studies have characterized the AOX genes as a potential source of polymorphisms. AOX polymorphisms have been highlighted, including intron length polymorphisms (ILPs) in *Hypericum perforatum* L. (St. John's Wort) (Ferreira et al. 2009), *Daucus carota* L. (Cardoso et al. 2009) and *Vitis vinifera* L. (Costa et al. 2009), retrotransposon integration in an intron of AOX2 in V. *vinifera* (Costa et al. 2009) and synonymous and non-synonymous SNPs in H. *perforatum* (Ferreira et al. 2009) and D. *carota* (Cardoso et al. 2009). AOX naturally occurring amino acid substitutions at Cys1 and/or Cys2 have been reported in rice (Oryza sativa; Ito et al. 1997), maize (*Zea mays*; Karpova et al. 2002) and in tomato (*Lycopersicon esculentum*; Holtzapffel et al. 2003).

Polymorphic sequences need to be importantly associated to a phenotype of interest in order to become a candidate for FM development. Experimental systems can be designed to pre-screen the importance of a gene and polymorphic site or pattern in physiological-biochemical assays. Appropriate experimental systems according to a specific trait of interest can be well defined under *in vitro* conditions to facilitate pre-screening in the breeding process. This approach makes sense in order to reduce the high costs and high time consumption of field testing (Arnholdt-Schmitt 2005a,b, Arnholdt-Schmitt et al. 2006).

A relationship between AOX regulation and interesting agronomic traits are indicated. The involvement of AOX in cell reprogramming was reported in embryogenic cells of D. *carota*. In the presence of the AOX inhibitor (SHAM), embryogenic cells were unable to develop embryogenic structures and its growth rate was diminished (Frederico et al.

2009). Sequence variations in two *AOX* genes of *Daucus carota* L. (*DcAOX2a* and *DcAOX2b*), putatively related to pre-miRNAs in intronic regions were found between genotypes (Cardoso et al. 2009, 2011). Involvement of *AOX* genes in stress responses on cell reprogramming was also demonstrated during the inoculation of differentiated secondary root phloem in a cytokinin-containing nutrient solution that induces tissue redifferentiation and callus growth (Campos et al. 2009).

As cited above much research has been performed on the characterization of the multigene 'alternative oxidase' (AOX) in diverse plant systems and in its relationship to adaptative cell reprogramming under stress. However, validation of the sequences as candidates for to FM development is still a challenge.

1.10.1. AOX and rooting: the hypothesis for FM development

Research has been conducted in order to determine differences in rooting potential of olive cultivars and clones (Aslmoshtaghi and Reza-Shahsavar 2009, Del Rio et al. 1991, Rahman et al. 2002, Bartolini et al. 2008). Several factors have been studied, like concentrations and pulse times of growth regulators (Epstein and Sagee 1992), environmental light and temperature (Hartmann and Loreti 1965, Morini et al. 1989, Fabbri et al. 2004), age of mother plant and nutritional status of the cuttings (Davies et al. 1982, Awang et al. 2011). Hereby achieved data solved most of the problems in commercial propagation for the easy-to-root olive cultivars. Unfortunately, this is not for the case of cv. Galega vulgar, which is hard to propagate vegetatively using semi-hardwood cuttings. As seen previously, this cultivar is very important in Portugal and especially appreciated due to its high quality regarding fruit taste and oil components. In this context, FM development can be an important tool when applied in olive nurseries for selection of “easier-to-root” genotypes.

The trait studied in the present thesis is the AR capacity under stress conditions generated for plant propagation. The AR formation has been proposed as an example of cell reprogramming upon stress (Arnholdt-Schmitt et al. 2006). Especially strong confirmation for the initiation of new developmental programmes through stress and/or treatments with growth regulators is coming from experience with *in vitro*

culture systems. Explantation and inoculation of plant material into a new chemical and physical surrounding is well-known to affect induction and initiation of new differential programmes, such as dedifferentiation, changes between heterotropic and autotropic growth as well as adventitious shoot and root growth, depending on the growth regulators supplied (Arnholdt-Schmitt et al. 1995a, Arnholdt-Schmitt et al. 1995b, De Klerk et al. 1997, Grieb et al. 1997, Arnholdt-Schmitt et al. 1999, Pasternak et al. 2002). Root initiation at shoot cutting is also an adventitious process. This means that root morphology is induced and initiated at a place, where, initially, there was no cell determination for roots in the developmental plan of the individual plant. So, it is possible to understand the reaction in olive shoots as response on an environment stress through cutting of the shoot and the additional inclusion into a solution with auxins.

Plant adaptation under stress condition is related to the capacity for plasticity. It is known as a main driver in evolution for organisms to occupy ecological niches, but has not been explored as a trait *per se* for molecular plant breeding (Cardoso and Arnholdt-Schmitt 2013).

Plant species exhibit higher or lower degree of plasticity. "Various studies suggest that species from more heterogeneous and changing environments have greater degrees of plasticity" (Nicotra et al. 2010). A variety of signaling cascades can be triggered in response to environmental signals. Subsequent genetic and epigenetic changes can occur in different cells/tissues, thus, altering gene expression differentially and thereby mediating plasticity. Among those changes, protein phosphorylation (Nüshe et al. 2007), histone modification, transposable element activation and jumping (Nicotra et al. 2010), alteration of DNA methylation patterns and small RNA activity have been shown to play critical roles in the response to abiotic stresses at both gene-specific and genome-wide level (Kapazoglou and Tsaftaris 2011).

One of the many biochemical responses to stress is the activation of the enzyme AOX, that have been suggested to play a significant role in early cell adaptation as also to growth and differentiation to different types of stress (Arnholdt-Schmitt et al. 2006).

Further, diverse mitochondrial dysfunctions associated with oxidative stress and resulted in the induction of AOX at transcript and protein level. For example: in

tomato, a chilling-tolerant genotype showed strong up-regulation of AOX in comparison to a chilling-sensitive genotype (Shi et al. 2013) and alteration in the AOX2 expression in soybean was observed on vegetative growth and seed yield under typical greenhouse growth conditions demonstrating benefit of sustaining plant vegetative growth and reproductive capacity (Chai et al. 2012). As a consequence, AOX is now often used as a general marker of mitochondrial dysfunction and/or cellular oxidative stress. Further, numerous abiotic and biotic stress conditions are known to elevate AOX amount, supporting the idea that such stresses impact mitochondrial function and that AOX induction might represent an important acclimation response (Vanlerberghe et al. 2013).

Earlier studies had already demonstrated physiological effects of stress. For example, phosphorus limitation in bean roots is accompanied by an increase of alternative respiration from 40-50% to 80-90% (Rynchter and Mikulska 1990). Also induction of new cell programs had been observed under oxygen stress (Pasternak et al. 2002) and others results pointed out an important additional function of AOX related to cell reprogramming apart from merely counteract oxidative stress (see Arnholdt-Schmitt et al. 2006). The hypothesis of the present work is based on the simple fact that stress-induced root initiation needs a change in substance and energy flow to build-up the new structures of emerging roots. Alternative respiration enables an increase in turnover rates of carbon skeletons and makes more flexible the energy metabolism related to cellular redox state under stress. Since AOX is the most important enzyme of this pathway, it can easily be suggested to play a key role in these metabolic imbalances (Clifton et al. 2005, Arnholdt-Schmitt et al. 2006).

Objectives of the study

The thesis has two general objectives. First, it was the aim to verify whether AOX is involved in the formation of adventitious roots in *Olea europaea* L..The second aim was to explore the availability of sequence polymorphisms in a selected AOX gene sequence as sources for functional marker development to assist future breeding on easy-to-root cultivars.

To achieve these general objectives the following specific tasks had been defined:

1. Isolation and sequence characterization of the multigene family alternative oxidase from *Olea europaea* L. (Chapter 2)

2. Demonstration of the critical involvement of AOX genes in the rooting process in the target tissues for rooting by applying the relevant commercial system conditions (Chapter 3 – this chapter shows also the complex research approach).

3. Selection of a primary candidate gene for the studies of the thesis and identification of promising AOX gene sequence polymorphisms for FM development
   (Chapter 3)

4. Adjustment of an *in vitro* microshoot culture system in order to facilitate studies on the functionality of AOX gene sequences in the rooting process. (Chapter 4)

5. Studying the involvement of AOX gene transcription in secondary metabolism linked to adventitious rooting under *in vitro* culture conditions (Chapter 5)

6. Developing perspectives for future experimentation to advance the initial studies of the complex research approach of the thesis (Chapter 6)

Thesis Format

This doctoral thesis was designed to be based on a group of manuscripts that are published and that will give body to the research of the thesis. As all articles were written to stand alone, the reader may find repetition in some parts of the manuscripts, especially in the introduction and method sections, as well as the final conclusion. This fact results from sharing the sample, instruments and procedures among the series of articles. All articles are somehow connected. This thesis was organized with an evident research rationale that connected all the articles in order to achieve the main goal of the work.

1.11. References

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Chapter 2 Isolation and Characterization of AOX genes in *Olea europaea* L.

2.1. Summary

Alternative Oxidase (AOX) is an inner mitochondrial membrane protein encoded by the nuclear genome. In higher plants, the multigene family encoding AOX consists of two subfamilies: *AOX1*-type and *AOX2*-type genes. In this chapter isolation and characterization of the *AOX* multigene family in *Olea europaea* L. is reported. Gene expansion was observed in only the *AOX1*-subfamily (*OeAOX1a*, *OeAOX1b* and *OeAOX1c*). From the *AOX2*-subfamily (*OeAOX2*) a single gene was identified. Complete open reading frames (ORFs) of the genes *OeAOX1a* and *OeAOX1c* and partial sequences of the genes *OeAOX1b* and *OeAOX2* could be isolated.

Sequence similarity in the ORFs of OeAOX1a and OeAOX1c were higher among AOX1 from other plants species than between both OeAOX1 sequences. In OeAOX1a the mitochondrial targeting sequence peptide, located at the N-terminal exon 1 region, could not be identified. It can be suspected that this gene might still be incomplete..
Gene sequence variation was investigated at the transcript level. Length and sequence heterogeneity was identified at the 3'-UTR of *OeAOX1a*. The influence of those polymorphisms on the plant phenotype is highlighted. A miRNA target site that was predicted in a polymorphic region can be explored in future for effects at the posttranscriptional level

2.2. Introduction

Development of FMs requires the identification of a target gene which could explain the phenotypic variation for a specific agronomic trait. Alternative oxidase (AOX), recently proposed as a functional marker (FM) for efficient cell reprogramming (Arnholdt-Schmitt et al. 2006, Cardoso and Arnholdt-Schmitt 2013), was selected for FM development to assist AR efficiency in olive.

Beside the selection of the target gene it is necessary to isolate the respective gene sequence or sequences of a gene family in order to search for polymorphisms which could be considered as candidates for FM development. It is known in higher plants, that *AOX* multigene family consists of genes belonging to two subfamilies termed *AOX1* and *AOX2* (Considine et al. 2002). The occurrence of those subfamilies species-dependent. To date, *AOX1* genes were found in monocots and eudicots, whereas *AOX2* genes were detected only in eudicot plant species (Considine et al. 2002) and in the gymnosperm *Pinus pinea* (Frederico et al. 2009b). In most of the species studied so far either the *AOX1* subgroup was expanded, as in the case of *Arabidopsis thaliana* (Saisho et al. 1997) or only the subgroup *AOX2*, as in the case of *Glycine max* or *Vigna unguiculata* (Costa et al. 2004). In *Daucus carota* a novel pattern of *AOX* sequences was described, showing a simultaneous occurrence of two gene sequences in both *AOX* subfamilies (Costa et al. 2009).

Conserved sequence differences between proteins encoded by both *AOX1* and *AOX2* gene subfamilies were identified that covered near-neighbor sequences of the CysI site (Costa et al. 2009, Frederico et al. 2009a). From *in silico* conservation analysis it is known that consideration of neighbor sequences can well-improve the prediction of conserved functional sites (Capra and Singh 2007) indicating the importance of such loci. A comparison performed across both *AOX* subgroup gene sequences from diverse plant species revealed the exon 3 as the most conserved region. This characteristic allowed Saisho et al. in 1997 to design degenerate primers (named P1 and P2), which were successfully applied across different plant species for *AOX* genes identification (e.g. Campos et al. 2009, Ferreira et al. 2009, Frederico et al. 2009a).

Among the procedure to characterize gene bodies, the most important but difficult to achieve work is the one related to the obtaining of the complete cDNA sequence. Several methods have been developed to facilitate the identification of the missing sequence and in 1988 Frohman et al. firstly proposed the *in vitro* RACE (*r*apid *a*mplification *c*DNA *e*nds) method. Several problems were identified and to overcome those Chenchick et al. (1996) published an improved RACE method based on the use of double strand adapters carefully designed to link in both cDNAs double strand (ds), followed by the amplification of 5’ and/or 3’RACE using a combination of gene specific

primer and adapter specific primer combined with the technology of long and accurate (LA)-PCR (Barnes 1994). With the Marathon^{TM} technology developed by Chenchik et al. (1996), after cDNA synthesis and adapter linkage to cDNAs ends, a library of non-cloned dscDNA is produced. The 5' and 3' RACE can be done for many genes using specific primers for the target gene. Within the methodologies available for cDNA ends isolation the RACE, based on PCR technique using cDNA as template, is known as the most efficient, fast and sensitive (Frohman et al. 1988). The successful use of the RACE methodology to isolate the 5' and 3' ends of *AOX* genes were already described for different plant species (Saisho et al. 1997, 2001, Polidoros et al. 2005, Campos et al. 2009).

Functional polymorphisms, which could occur in both protein-coding and non-coding regions, could affect gene structures and expression patterns, and may consequently, contribute to phenotypic variations. Several functional polymorphisms have been identified at the promoter region in human genes (Le Goff et al. 2002) and also in crop plants (Su et al. 2011). Nevertheless, the most part of the successfully developed FMs so far for plant breeding application are based on polymorphisms located at the gene body (see review at Cardoso and Arnholdt-Schmitt 2013). They are mostly associated with non-synonymous single nucleotide polymorphisms (nsSNPs) (Bradbury et al. 2005a,b, Shi et al. 2008, Lagudah et al. 2009, Li et al. 2009) and few associated with SNPs located in the intronic regions (Frary et al. 2000, Smýkal et al. 2010).

Polymorphisms occurring in protein-coding sequences can affect the phenotype by changing the protein sequence and consequently protein structure and function, or could interrupt the protein sequence due to a nonsense mutation creating a truncated protein with consequent loss-of-function. In introns, polymorphisms can be functionally critical in view of its potential to influence binding of transcription factors (Xie et al. 2005), alternative splicing (Baek et al. 2008), the coding of intronic regulatory elements, such as micro- or small nucleolar- RNAs (Li et al. 2007), as well as nonsense-mediated mRNA decay (Jaillon et al. 2008). Polymorphisms located at protein coding and non-coding sequences were already described in *AOX* genes for different plant species (Cardoso et al. 2009, 2011, Ferreira et al. 2009, Frederico et al. 2009a),

however, the data available are still limited to establish an unequivocal link between AOX sequence polymorphism and phenotypic variation. Nevertheless, a mutagenesis experiment performed in rice demonstrated the functionality of nsSNP related to low temperature tolerance (Abe et al. 2002).

Beside the polymorphisms identified in *AOX* considering the analysis of genomic DNA, another aspect of polymorphism in AOX sequences is the 3'-UTR microheterogeneity described in maize by the appearance of transcripts with different 3'-UTR lengths, as a result of alternative polyadenylation (Polidoros et al. 2005). There are many processes of post-transcriptional control of gene expression involving the 3'-UTR and polyadenylation signal of the gene. The nature of the 3'-UTR and the choice of polyadenylation site in genes with multiple sites may play a role in the expression of a gene, with important physiological consequences, which in breeding could correspond to a change at the target agronomic trait. Several examples of regulatory elements residing in 3'-UTRs were well described by Polidoros et al. (2009).

Polymorphisms could have an effect on both *AOX* expression and AOX function due by several different mechanisms. From the perspective of olive breeding, it will be important if will be possible to identify AOX polymorphic sequences that can later be shown to be closely associated to AR efficiency.

2.3. Material and Methods

2.3.1. Isolation of *Olea europaea* L. *AOX* genes (*OeAOX*)

Plant material and DNA Extraction

Young leaves (± 100 mg) of four *in vitro* cultured microshoots of *Olea europaea* cv. Galega vulgar, previously established *in vitro* according to Peixe et al. (2007), were used for genomic DNA (gDNA) extraction using the DNEasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. gDNA integrity analysis and quantification was performed by electrophoresis in 1% agarose gel (Invitrogen Life technologies, UK) after staining in ethidium bromide (EtBr) (Merck, Germany) solution (2 ng/ ml). Quantification was performed in comparison with defined amounts of *λ*

DNA (Fermentas, EU). Gel documentation was performed with the Gene Flash Bio Imaging System (Syngene, Cambridge, UK).

PCR conditions

To search for *AOX* genes, PCRs were carried out using 10 ng of gDNA as template in a reaction volume of 50 µl. Two combinations of degenerate primers were tested in order to isolate a partial sequence of exon 3 (if considering the common structure of *AOX* plant genes with four exons and three introns) 42AOXF: 5'-GCDGCDGTBCCDGGVATGGT-3'/45AOXR:5'-TCVCKRTGRTGHGCYTCRTC-3' (Hélio Costa et al., personal communication) and P1/P2 (Saisho et al. 1997) (see primers location in the scheme of Fig. 1).

PCR was carried out for 35 cycles in the 2720 Thermocycler (Applied Biosystems, Singapore). Each cycle consisted in 60 s at 95 ^{o}C for denaturation, 2 min at 50 ^{o}C for primers annealing and 2 min at 72 ^{o}C for DNA synthesis. An initial step at 95 ^{o}C for 5 min and a final step at 72 ^{o}C for 5 min were included. For the PCR a mix of 0.5 U of *Taq* DNA polymerase (Fermentas, Ontario, Canada) were used with 1x manufacturer supplied $(SO_4)NH_4$ buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate (dNTPs) (Fermentas, Ontario, Canada) and 0.2 µM of each primer. PCR products were analyzed by electrophoresis in 1.4% (w/v) agarose gel (Invitrogen Life technologies, UK) following the procedure described above.

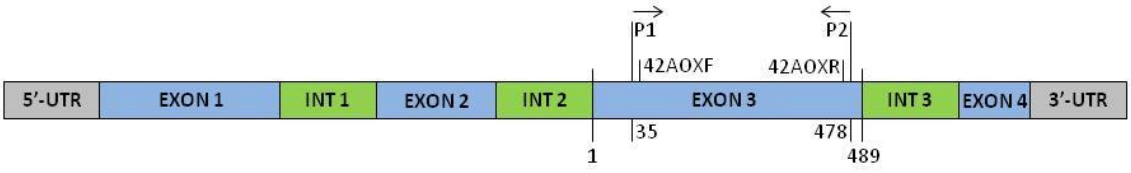

Fig. 1. Scheme representing the common structure of *AOX* genes with four exons and three introns. The numbers 1 and 489 indicate the complete size of exon 3 in the *AOX* genes with the conserved structure. The numbers 35 and 478 indicate the location of primers P1 and P2, respectively.

2.3.2. Isolation of *OeAOX* genes ends

Plant material, RNA Extraction and cDNA synthesis

Total RNA was extracted from a bulked sample of 10 *in vitro* cultured microshoots of *O. europaea* cv. Galega vulgar, using Rneasy Plant Plant Mini Kit (20) (Qiagen, Germany), with on-column digestion of DNA with the RNase-Free DNase Set (Qiagen, Germany) according to the manufacture's protocol. The RNA integrity was evaluated in 1.4% diethyl pyrocarbonate (DEPC, Sigma, St. Louis, MO, USA) agarose gel and visualized as described in 2.3.1. RNA quantification was performed in a spectrophotometer (DU 530, Beckman Coulter Inc., Fullerton, CA, USA).

5'-Rapid Amplification cDNA Ends (5'-RACE)

For the 5' end isolation 1 μg of total RNA was reverse transcribed using the FirstChoice RLM-RACE Kit (Ambion, Austin, TX, USA), following the manufacture's protocol specific for 5'-RACE. After cDNA synthesis, two PCRs were performed for each gene using Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England). The first PCR was performed using as template 1 μl of cDNA. For the second PCR 1 μl of the first PCR product was used as template. The primers used in each reaction are described in Table 1.

Table 1: Specific reverse primer sequences used in the isolation of the 5'-end of the *AOX1a* and *AOX1c* genes in *Olea europaea* L (*OeAOX1a* and *OeAOX1c*) and PCR conditions. Forward primers were provided by the First Choice RLM-RACE Kit (Ambion, Austin, TX, USA).

PCR	Gene	Reverse primer	PCR conditions
1	***OeAOX1a*** ***OeAOX1c***	5ROlea Out: 5'-MGCMGGWMYATTYTCAAYARKG-3'	30 s at 94 °C 60 s at 50 °C 2 min at 72 °C
2	***OeAOX1a***	5ROlea Inta: 5'-GCATTGAAAAATACGCCCTGC-3'	30 s at 94 °C 60 s at 50 °C 2 min at 72 °C
	OeAOX1c	5ROlea Intc: 5'-GCATTTGCAAATACACCTTGG-3'	30 s at 94 °C 60 s at 58 °C 2 min at 72°C

PCRs were carried out with an initial denaturation at 94^{o}C for 5 min followed by 35 cycles with the conditions defined in Table 1. A final extension at 72^{o}C for 10 min was also performed. The result of the second PCR was analyzed by electrophoresis in 1.4% agarose gel following the procedure previously described.

3'-Rapid Amplification cDNA Ends (3'-RACE)

Total RNA was reverse-transcribed using 5 µg of total RNA and 2 µM of the oligo (dT) 16 primer VIAL 8: 5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV (V=A,C ou G)-3' (Roche, Mannheim, Germany). Reaction mixture was incubated for 5 min at 70 ^{o}C, and after placed on ice for 5 min. Following, the remain reagents were added to the previous mixture for a final volume of 50 µL: 1x enzime buffer *RevertAid^{Tm} HMinus M-MuLV Reverse* (Fermentas, Ontario, Canada), 1 mg/ml of BSA (Ambion), 1 mM each dNTP (Fermentas, Ontario, Canada), 0.05 µg/µl of Actinomycin D (Roche, Mannheim, Germany), 2 mM of dithiothreitol (DTT) (Fermentas, Ontario, Canada), 0.8 U/µl of RNase inhibitor (Fermentas, Ontario, Canada) and 6 U/µl of the enzyme *RevertAid^{Tm} HMinus M-MuLV Reverse* (Fermentas, Ontario, Canada). This reaction mixture was incubated for 90 min at 42 ^{o}C. The cDNA product was stored at -20 ^{o}C.

In case of *OeAOX1a* and *OeAOX1c* PCR was carried out, for a final volume of 50 µl, using 1 µl of cDNA as template, 0.5 U of *Taq* DNA polymerase (Fermentas, Ontario, Canada), 1x $(NH_4)_2SO_4$ buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP and 0.2 µM of each primer. The VIAL 9 (5'-GACCACGCGTATCGATGTCGAC-3', Roche, Mannheim, Germany), complementary reverse primer to VIAL 8 was used in combination with each forward primer. PCRs were carried out following the conditions described in Table 2.

Table 2: Specific forward primers used in combination with VIAL 9 for the isolation of the 3'-end of the *AOX1a, AOX1c and AOX2* genes in *Olea europaea* L. and respective amplification programme. The reverse primer used in combination was in all cases the VIAL 9 (Roche, Mannheim, Germany).

Gene	Forward primer	PCR conditions
OeAOX1a	P1: 5'-CTGTAGCAGCAGTVCCTGGVATGGT-3'	30 s at 94 °C 30 s at 60 °C 60 s at 72 °C
OeAOX1c	3RoleaD: 5'-CTCATCGCATAGTAGGCTACC-3'	30 s at 94 °C 30 s at 60 °C 60 s at 72 °C
OeAOX2	OeAOX2_33fw: 5'- ATTGCATCTCAGGTCTCTTCGC-3'	30 s at 94 °C 90 s at 68 °C

In case of *OeAOX2* the PCR was performed in 25 µl volume using Taq Ready-to-go PCR Beads (GE Healthcare, Little Chalfont, England), adding 1 µl of cDNA as template and 0.2 mM of each primer. The forward primer sequences and PCR program are described on Table2. PCR products were visualized in 1.4% agarose gel electrophoresis following the procedure previously described.

2.3.3. Cloning and sequence analysis

Amplicons generated by the different PCRs were gel-purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and separately cloned into a bacterial plasmid pGem®-T Easy vector (Promega, USA), transformed into *Escherichia coli* competent cells strain JM109 (Promega, USA), and selected according to the manufacturer's instructions. Plasmid DNA was extracted according to the alkaline lyses protocol (Birnboim and Doly 1979) and firstly analyzed with the restriction enzyme *Eco*RI (Fermentas, Ontario, Canada) in order to select the clones carrying the amplicon with the right length. Plasmid DNA of selected clones was analyzed by using the enzymes *Hpy*F3I*, Alu*I and *Bsp*143I (Fermentas, Ontario, Canada) in order to identify different restriction patterns due to differences at the gene sequence. Restriction reactions were carried out separately according to manufacturer's protocol, with exception of *Hpy*F3I and *Alu*I, which were used together. Products of restriction reactions were analyzed in 1.4% (w/v) agarose gel (Invitrogen Life technologies, UK) as

previously described, and clones showing different restriction patterns were selected to sequencing.

Plasmid DNA from all selected clones were sequenced by commercial services through the company Macrogen (www.macrogen.com). All sequences resulted from at least two sequencing cycles, in sense and antisense directions, using specific primers located within the vector (T7 and SP6, Promega, Madison, WI).

Sequence homology was searched in the NCBI data basis (National Center for Biotechnology Information, Bethesda, MD) using BLAST algorithm (Karlin and Altschul 1993) (http://www.ncbi.nlm.nih.gov/) (BLASTn and BLASTp). Amplicon sequences giving high homologies with plant *AOX* genes were translated into peptide sequences using EditSeq from Lasergene 7 software (GATC Biotech, Konstanz). Sequences alignments were performed by multiple alignment clustal W using the MegAlign software from Lasergene 7 (GATC Biotech, Konstanz). Complete gene sequences of *OeAOX1a* and *OeAOX2* were *in silico* translated to amino acids sequence using the EditSeq software from LASERGENE 7 (GATC Biotech, Konstanz). Partial sequences (from 5' and 3'-RACE and from the cloning of the internal partial gene sequence) were analyzed with SeqMan software from LASERGENE 7 (GATC Biotech, Konstanz), in order to *in silico* construct the complete genes sequences.

Phylogenetic studies included AOX sequences available in NCBI data basis and olive sequences were performed using an Clustal W Multiple alignment performed with BIOEDIT software (Hall 1999). The alignments were bootstrapped with 1000 replicates by the Neighbor-Joining method using the MEGA 4 software (Tamura et al. 2007).

MitoProt II 1.0 software (Claros and Vincens 1996) (http://ihg.gsf.de/ihg/mitoprot.html) was used to predict the mitochondrial targeting sequence cleavage site.

Putative miRNA target binding sites were predicted using psRNATarget (Dai et al. 2011). Default settings were used with the exception of expectation values which were set to 4.0 in order to increase the prediction coverage.

2.4. Results

PCR amplification of AOX fragments

To identify the number *AOX* genes in *Olea europaea* the PCR was carried out with two different pairs of degenerate primers using gDNA as template. From the amplification carried out with the primers 42AOXF/45AOXR was possible to obtain a single band with approximately 450 bp (Fig. 2A). A fragment with similar size was also obtained using the primers P1/P2 (Fig. 2B), which was expected according the results provided by Saisho et al. (1997) in *AOX* genes isolation from *Arabidopsis thaliana*. Both fragments were independently cloned and plasmid DNA from 16 clones was analyzed from 42AOXF/45AOXR and from 28 clones from P1/P2. The analysis made by restriction enzymes allowed selection of 8 bacterial clones from the amplicon provided by amplification with 42AOXF/45AOXR (see example of clones screening in Fig. 3) and 7 clones from the amplicon provided from amplification with P1/P2.

The identification of the olive *AOX* sequences was based in the high homology with *AOX* gene sequences from other plant species available at NCBI data basis. Nucleotide sequences from six clones of P1/P2 primers combination (1, 2, 3, 7, 10 and 15), all with 444 bp length, presented between 76 and 95% homology with *AOX* from different plant species. Clones 1, 2, 3 and 15 presented the highest homology with *AOX1* of *Pinus pinea* (82-83%, acc. GQ380552.1), clones 7 with *AOX1c* of *Hypericum perforatum* (95%, acc. EU330416.1), and clone 10 with *AOX1b* of *Solanum lycopersicum* (76%, acc. NM_001247191.1).

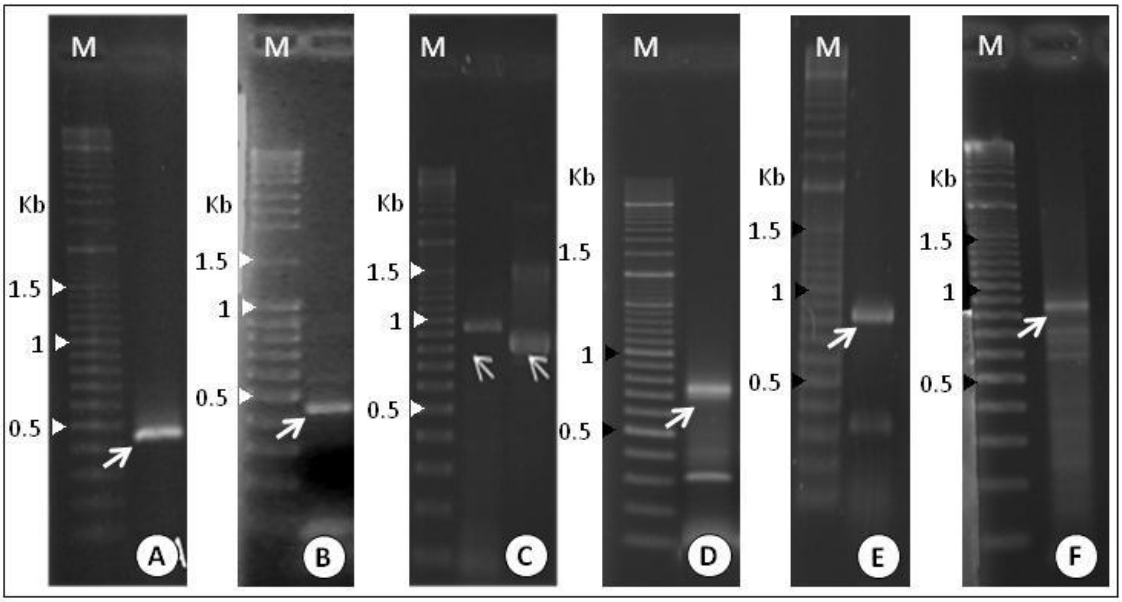

Fig. 2. Photograph of agarose gels showing the results of the different amplifications performed in order to identify the different AOX genes and for gene ends isolation. (A) amplification using the primers 42AOXF/45AOXR, (B) using the primers P1/P2, (C) 5'RACE for *OeAOX1a* in the second line and *OeAOX1c* in the third line, (D) 3'RACE using the primer forward P1, (E) 3'RACE using the primer forward 3RoleaD, (F) 3'RACE using the primer forward OeAOX2_33fw. The selected bands for amplicon cloning are indicated by arrow. M: MassRuler™ DNA Ladder (Fermentas, Ontario, Canada).

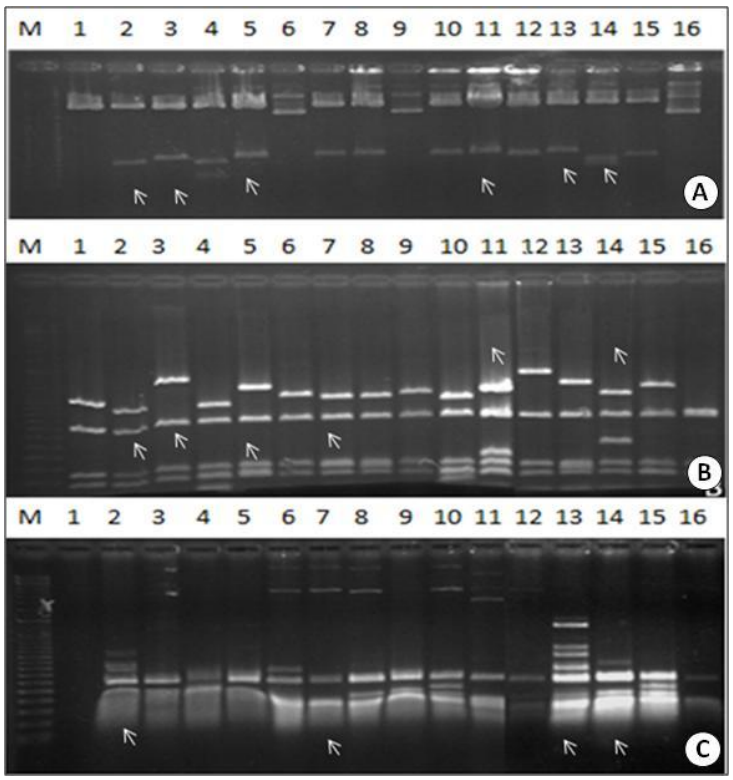

Fig. 3. Photograph of agarose gels showing the results of the restriction analysis of 16 plasmidic DNA with the 42AOXF/45AOXR amplicon using different restriction enzymes. (A) *Eco*RI, (B) *Hpy*F3I + *Alu*I, (C) *Bsp*143I. M: MassRuler™ DNA Ladder (Fermentas, Ontario, Canada). Arrows indicate the clones selected for sequencing.

The alignment of the six P1/P2 sequences shows high similarity among sequences of clones 1, 2, 3 and 15, and high similarity between sequences of clones 7 and 10 (Fig. 4). The 16 polymorphic sites indicate the existence of some SNPs, however a more detailed study is required. Usually, the SNPs located at the annealing region of degenerate primers are false SNPs; among clones 1, 2, 3 and 15, 4 SNPs are located on those regions. In other way, also clones 7 and 10 have higher similarity between them with only 5 nucleotide changes, from which 4 are located at the degenerate primer annealing region. Those differences decrease when the comparison is made among the deduced peptide sequences (Fig. 5).

The BLAST performed using the peptide sequences showed homologies between 88-100% with AOX sequences from other plant species. Sequences from clones 1, 2, 3 and 15 showed the highest homology with AOX1b of *Daucus carota* (between 97-100%, acc. ABZ81228.2) and from clones 7 and 10 with AOX1b of *Solanum tuberosum* (88%, acc. NP_001234120.1).

From the eight clones of 42AOXF/45AOXR only two nucleotide sequences (each with 431 bp size) showed homology with *AOX* genes available at the NCBI data basis. Sequence of clone 3 showed high homology with the *AOX2* of *Vitis vinifera* (99%, acc. EU165192.1), and of clone 5 with *AOX1a* of *P. pinea* (97%, GQ380556.1). The search for homology using the amino acid deduced sequences showed high homology of clone 3 sequence with AOX2 of *V. vinifera* (100% with acc. ACI28866.1 and 94% with ACB45425.1) and of clone 5 with AOX1a of *Pinus pinea* (97%, ACV60633.1). The analysis of the deduced peptide sequences shows differences between both sequences and also between these two and the other sequences identified with P1/P2 primers (Fig. 5).

From the interpretation of the dendrogram, constructed using the eight clones translated sequences identified as being *AOX* and considering the common region between all sequences, which means 143 amino acids, it is possible to define the existence of four different AOX peptide sequences, three codified by genes belonging to *AOX1*-subfamily and one to the *AOX2*-subfamily (Fig. 6). The *AOX1*-subfamily is represented by the named *OeAOX1a* (sequences of clones P1P2_cl1, cl2, cl3 and cl15),

OeAOX1b (sequence of clone 4245_cl5) and the *OeAOX1c* (P1P2_cl7); the *AOX2*-subfamily is represented by a single gene (4245_cl3).

In the alignment performed for the dendrogram construction it was possible to confirm that all the isolated sequences align with exon 3 sequences from genes that are known to have a structure consisting of four exons.

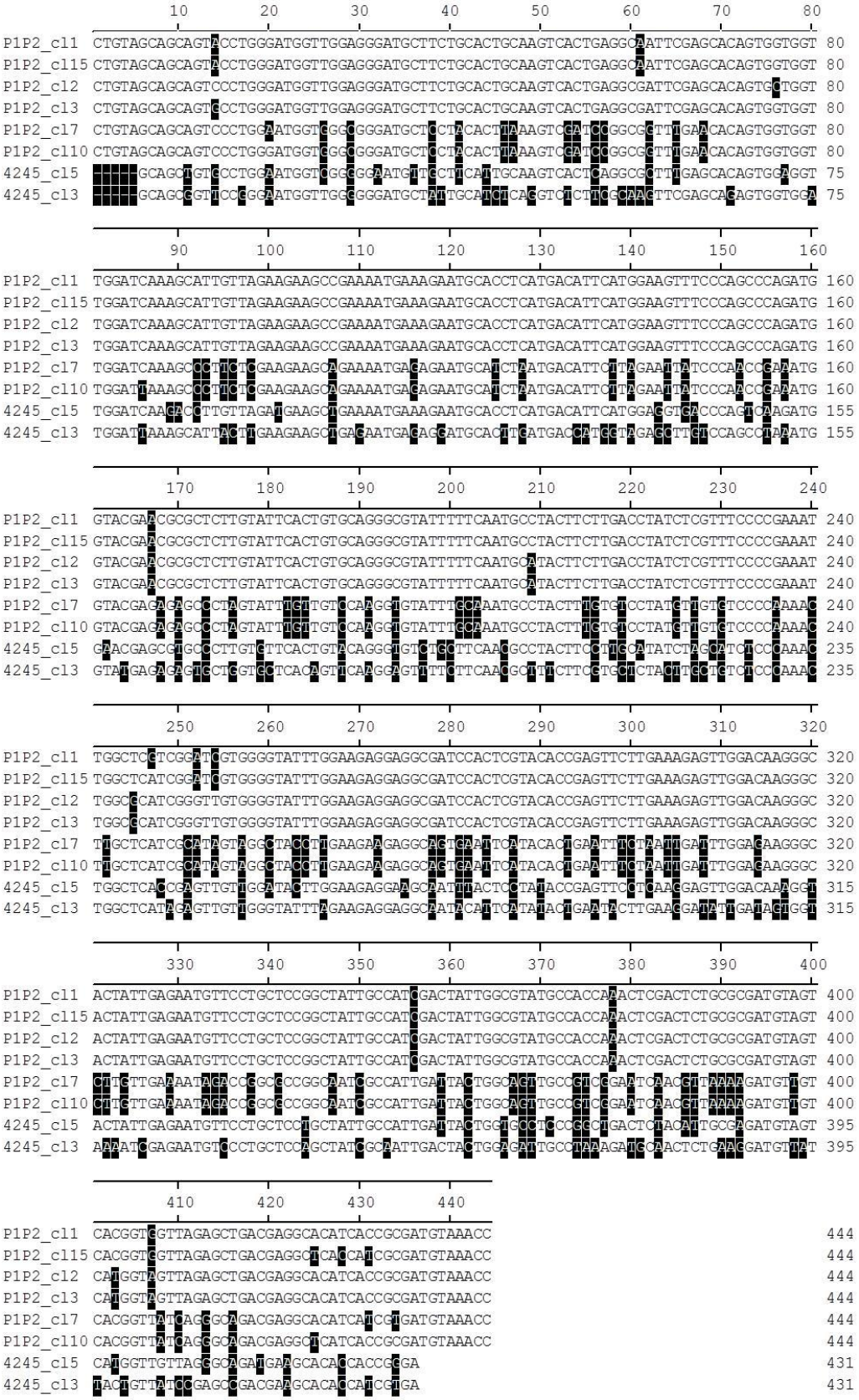

Fig. 4. Aligment of nucleotide sequences isolated with P1/P2 (clones 1, 2, 3, 7, 10 and 15) and 42/45 (clones 3 and 5) primers combinations. In black are the sites differing between sequences. Note that the sequences isolated with 42/45 primers combination are 5 bp shorter at 5' and 8 bp shorter at 3' when compared with sequences isolated with P1/P2 primers pair.

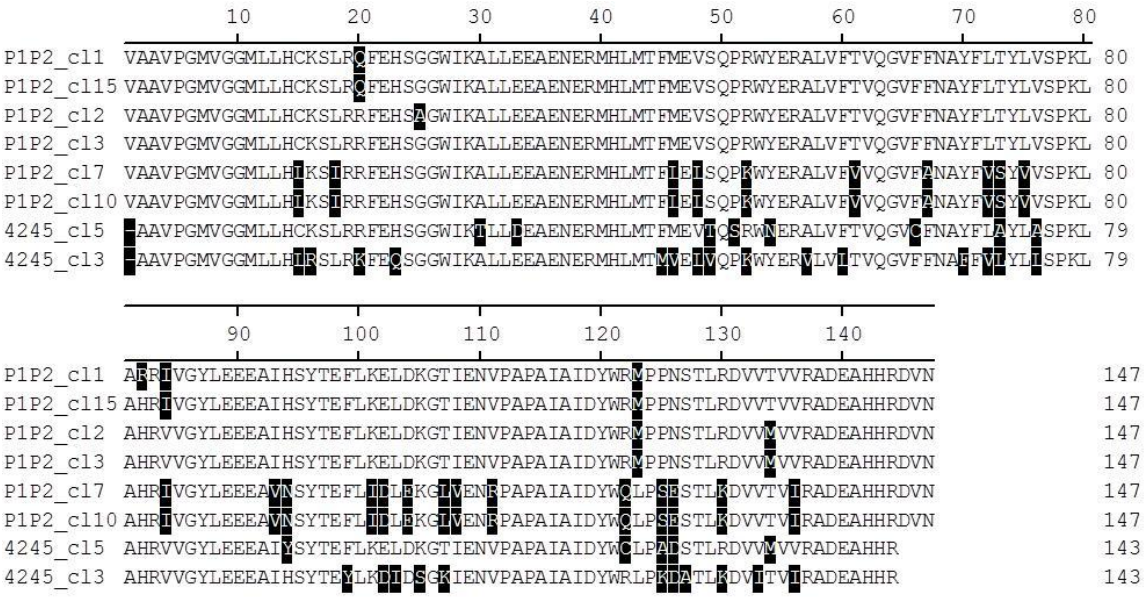

Fig. 5. Aligment of amino acid sequences deduced from the nucleotide sequences isolated with P1/P2 (clones 1, 2, 3, 7, 10 and 15) and 42/45 (clones 3 and 5) primers combination. In black are the amino acids differing between sequences. At the amino acids sequence level sequence P1P2_cl7 is equal to P1P2_cl10. Note that the sequences isolated with 42/45 primers combination are 1 amino acid shorter at 5' and 3 amino acid shorter at 3' when compared with sequences isolated with P1/P2 primers pair. The common region of 143 amino acids were considered for dendrogram construction at the figure below.

Isolation of AOX genes ends

5'-RACE

Through the isolation of exon 3 sequences belonging to four different genes it was possible to designing forward and reverse specific primers in order to isolate the 3' and 5' end, respectively. For 5' end isolation, the use of two reverse specific primers (Table 1) in combination with the primers supplied by the kit FirstChoice RLM-RACE Kit (Ambion, Austin, TX, USA) allowed the isolation of a fragment near 950 bp for *OeAOX1a* and 800 bp for *OeAOX1c*. After plasmid DNA extraction, screening with the *Eco*RI enzyme and sequencing, sequence analysis revealed 3 clones of *OeAOX1a* 5'-RACE (clones 1, 2 and 7) and 2 clones of *OeAOX1c* (clones 3 and 4) with homology with AOX plant genes. The match of those sequences with the exon 3 partial sequences

previously isolated allowed having the complete 5' end of both *OeAOX1a* and *OeAOX1c* genes.

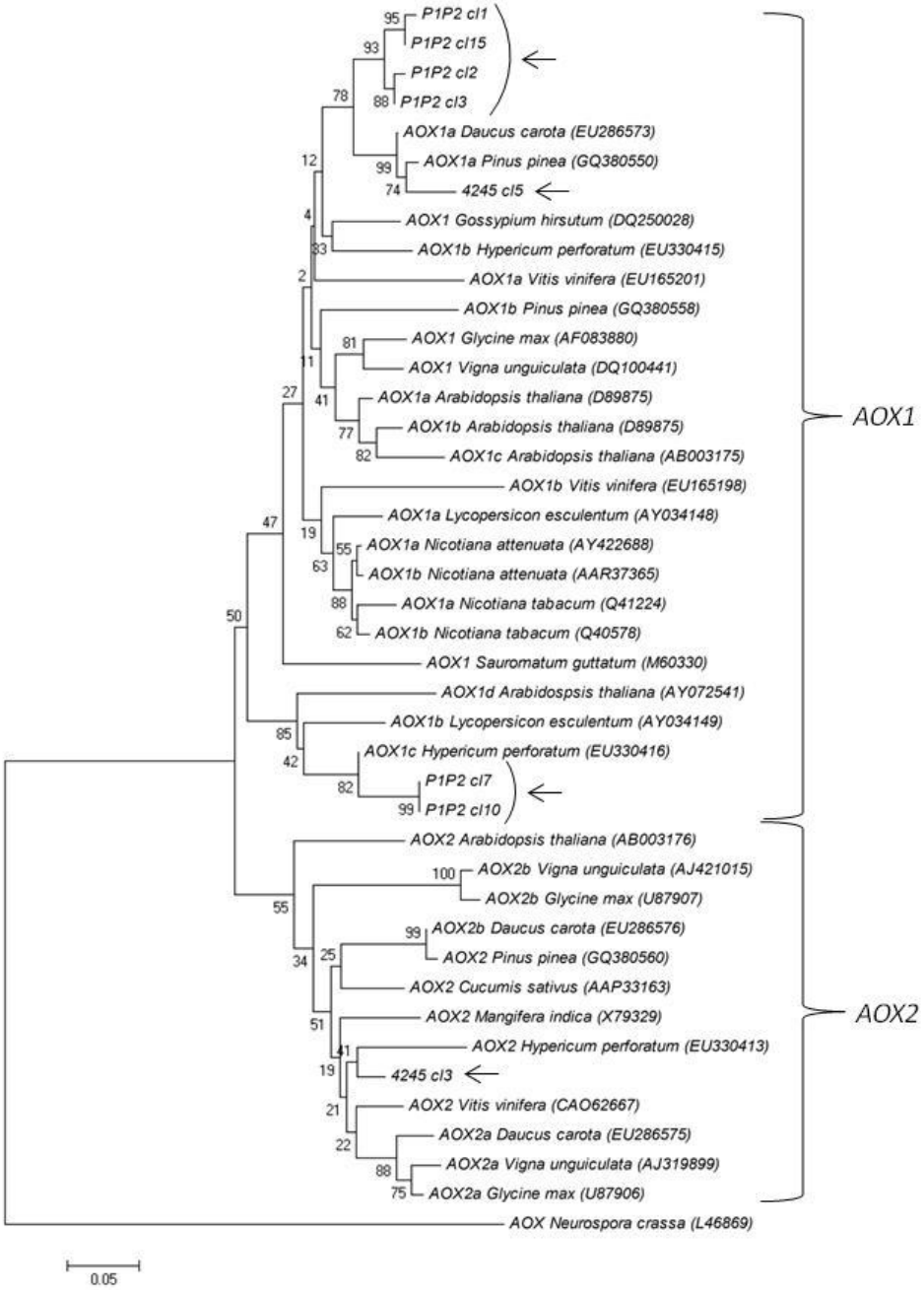

Fig. 6. Dendrogram describing the relationship among AOX proteins from higher plants, including the eight AOX clone sequences of *O. europaea*. The alignment of 143 amino acids was bootstrapped with 1000 replicates by the Neigbor-Joining method using the MEGA 4 software. The fungus AOX *Neurospora crassa* was used as outgroup. The scale bar indicates the relative amount of change along branches.

3'-RACE

In relation to the 3' ends isolation, the use of the forward primers specific for the *OeAOX1a*, *OeAOX1c* and *OeAOX2* (see Table 2) in combination with Vial 9, allowed the amplification of fragments with ± 950, 800 and 750 bp (see Fig. 2D, 2E and 2F). After cloning, plasmid DNA was extracted and following analyzed using *EcoR*I enzyme. The enzymes *HpyF*3I, *Alu*I and *Bsp*143I were also used to search for polymorphic sequences.

From selected clones 11 corresponding to *OeAOX1a*, 2 clones of *OeAOX1c* and 9 clones of *OeAOX2* showed homology with *AOX*. In the case of *OeAOX1a* and *OeAOX2* genes a high variability among the sequences of the 3' end was identified. In the following the variability at the 3'-UTR of *OeAOX1a* will be analyzed. The data for *OeAOX2* will be well described in chapter 3.

3'-UTR sequence and length variability in transcripts of *OeAOX1a*

Transcript analyses of the *OeAOX1a* were performed from a bulked sample of 10 *in vitro* cultured microshoots of cv. Galega vulgar. Table 3 shows the different lengths identified at the 3'-UTR, which range between 160 and 245 bp, and the correspondent number of clones detected for each 3'-UTR size (see alignment in Fig. 7).

Table 3. Alternative polyadenylation of the *OeAOX1a* transcripts ('Galega vulgar') detected by 3'-RACE experiments. The length of the 3'-UTR in bases, the number of clones detected for each 3'-UTR size and the nomenclature of the sequence are shown.

3'-UTR size (bp)	Number of clones	Sequence identification
160	1	Gal_1
179	3	Gal_2
184	1	Gal_3
193	1	Gal_4
221	2	Gal_5
222	1	Gal_7
223	1	Gal_6
245	1	Gal_8

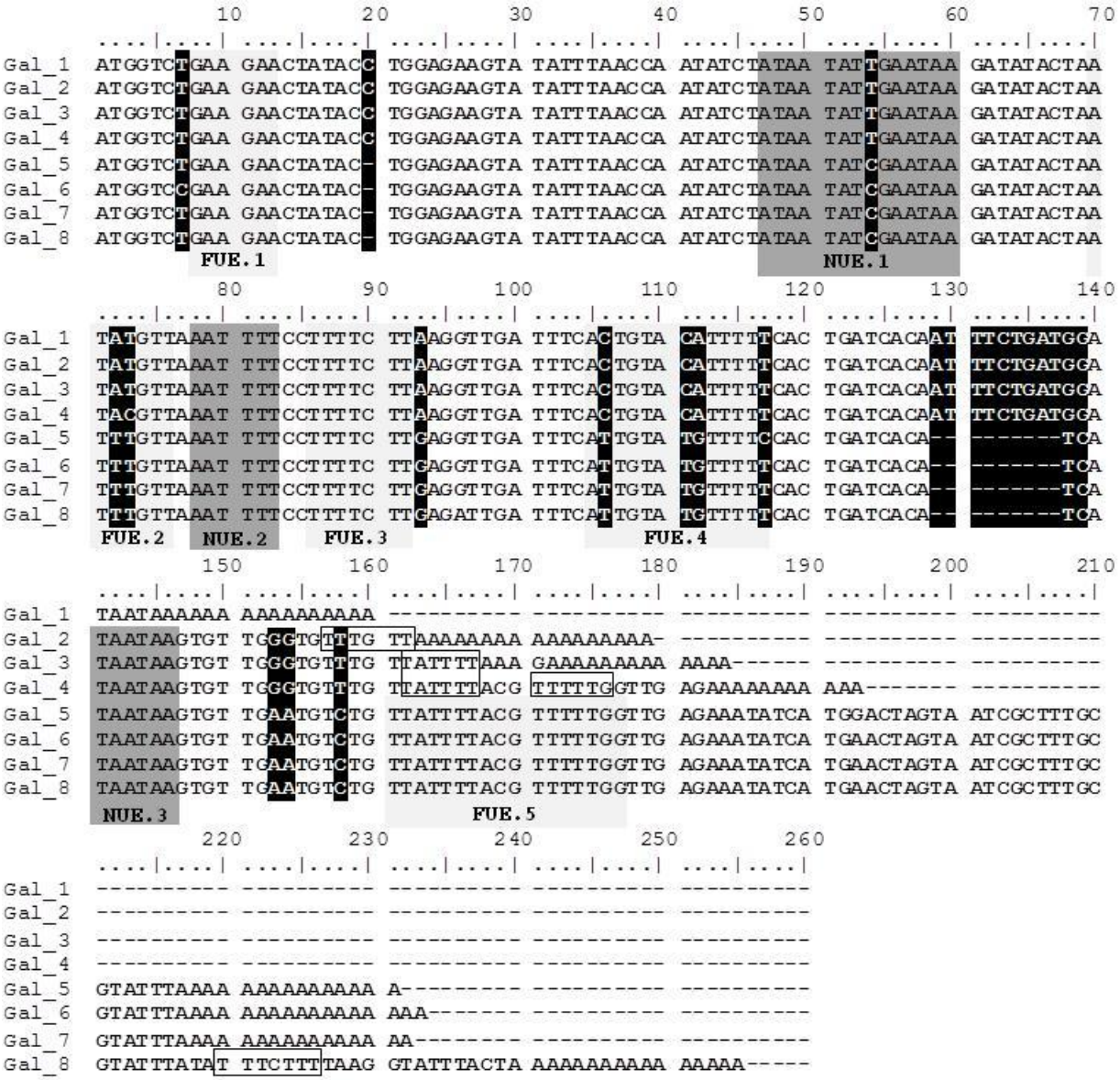

Fig. 7. Alignment of the 3'-UTR sequences identified in eight different *OeAOX1a* transcripts of *O. europaea* cv. Galega vulgar. Nucleotides positions which are not similar appear in black in the alignment.

It is known that 3'-UTR plays an important regulatory role during eukaryotic gene expression which makes interesting it to search for polymorphism at that gene region. Three major groups of poly (A) signals were identified in plants, corresponding to the near upstream element (NUE), far upstream element (FUE) and cleavage element (CE) (Fig. 8, see more detailed description on Chapter 3). Analysis of 3'-UTR sequences of *OeAOX1a* allowed to the identification of different putative positions for the three polyadenylation signals, which were already reported in *Arabidopsis thaliana* and *Oryza sativa* (Loke et al. 2005, Shen et al. 2008) and also identified in *OeAOX2a*, which

is well described in Chapter. 3. Despite the inexistence of the sequence AAUAAA, known as the most frequent signal for NUE in animals and plants, there was possible to identify several other sequences described as FUE and NUE signals (see Fig. 7). Some signal sequences were identified in polymorphic regions being signals for polyadenylation not in all transcripts (e.g. FUE 2 sequence is ATTTGTT, which is only present in transcripts of clones 5-8, due to the SNP_{72}C/T).

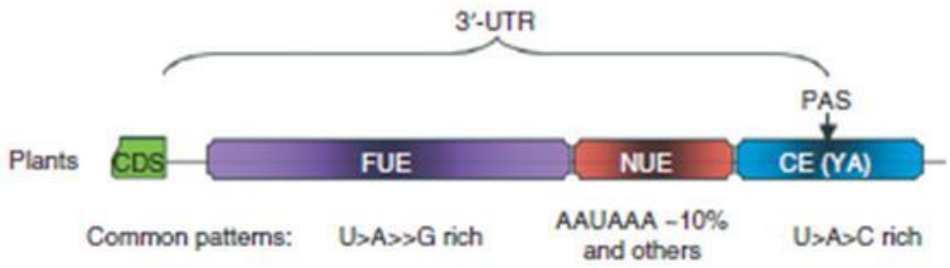

Fig. 8. Polyadenylation signals of nuclear mRNA in plants. CDS, protein coding sequence; UTR, untranslated region; FUE, far upstream element; NUE, near upstream element; CE, cleavage element; PAS, poly(A) site; YA, predominant dinucleotide located at the poly(A) or cleavage site where Y = U or C. The 'A' is the last nucleotide before poly(A) tail; > or >> indicates that one nucleotide appears more than another. Not drawn to scale. Adaptated from Xing and Li (2011).

Post-translational regulation of mRNA due by the existence of target sequences for miRNA binding is nowadays well known. In plants, beside the location of miRNA target binding sites on the protein coding sequence, it can be also found in the 3'-UTR (Rhoades et al. 2002). Analysis of *OeAOX1a* sequences allowed the identification of one putative target binding site on a polymorphic region, being detected only in Gal_5, _6, _7 and _8 (see sequence on Table 4). Searching for miRNA which could be homologous in the correspondent miRNA in olive identified the gma-MIR1526 in *Glycine max* (Subramanian et al. 2008).

Table 4. miRNA target binding site predicted at the 3'-UTR of the sequences Gal_5, _6, _7 and _8.

miRNA identification	nt position	Alignment	UPE
Gma-MIR1526	71-92	Query: 3' AACGAAUUAAAAGGAGAAGGCC 5' ::::.: ::::::::::.:::. : Ref: 5' UUGUUAAAUUUUCCUUUUCUUG 3'	14.221

UPE: target accessibility

Analysis of complete *OeAOX* gene sequences

Work performed allowed to the isolation of two complete AOX gene sequences (*OeAOX1a* and *OeAOX1c*) and two partial gene sequences, one already complete at 3' end (*OeAOX2*). Figures 9, 10, 11 and 12 show the nucleotide sequences and the putative corresponding proteins for *OeAOX1a*, *OeAOX1b* (Acc. No JX912720), *OeAOX1c* (Acc. No JX912721) and *OeAOX2* (Acc. No JX912722). The ATG in the beginning of the open reading frame (ORF) of both genes is the correct start of translation, because it is the first start codon resulting in an open reading frame, and stop codons are present in all three open reading frames of the transcript before this ATG.

The ORF length of *OeAOX1c* gene is 1062 bp resulting in a deduced protein with 353 amino acids length, which is similar with other plant *AOX* genes available at the NCBI data basis showing protein size always higher than 300 amino acids. In case of *OeAOX1a* the ORF length is 792 bp resulting in a shorter deduced protein sequence of 263 amino acids.

```
TGAATGAACACTGCGTTTGCTGGCAATAATAACAAGGGAATAGTGAGTTATTGGGGCGTAGAGCCTGCCA 70
   M  N  T  A  F  A  G  N  N  N  K  G  I  V  S  Y  W  G  V  E  P  A

AGATTACTAAAGAGGATGGCTCTGAATGGAGGTGGAACTGCTTTAAGCCATGGGAGACATACAAGACTGA 140
K  I  T  K  E  D  G  S  E  W  R  W  N  C  F  K  P  W  E  T  Y  K  T  D

TCTGTCTATAGATCTGAAGAAACACCACGCCCCTGTCACATTCTTAGACAAGGTGGCATATTGGACCGTC 210
  L  S  I  D  L  K  K  H  H  A  P  V  T  F  L  D  K  V  A  Y  W  T  V

AAGTCTCTCAGATTTCCTACAGATATATTCTTTCAGAGGCGGTATGGATGTCGTGCTATGATGCTGGAAA 280
 K  S  L  R  F  P  T  D  I  F  F  Q  R  R  Y  G  C  R  A  M  M  L  E

CTGTGGCCGCTGTGCCTGGCATGATTGGAGGGATGCTTCTGCACTGCAAGTCACTGAGGCGATTCGAGCA 350
T  V  A  A  V  P  G  M  I  G  G  M  L  L  H  C  K  S  L  R  R  F  E  H

CAGTGGTGGTTGGATCAAAGCATTGTTAGAAGAAGCCGAAAATGAAAGAATGCACCTCATGACATTCATG 420
  S  G  G  W  I  K  A  L  L  E  E  A  E  N  E  R  M  H  L  M  T  F  M

GAAGTTTCCCAGCCCAGATGGTACGAACGCGCTCTTGTATTCACTGTGCAGGGCGTATTTTTCAATGCAT 490
 E  V  S  Q  P  R  W  Y  E  R  A  L  V  F  T  V  Q  G  V  F  F  N  A

ACTTCTTGACCTATCTCGTTTCCCCGAAATTGGCGCATCGGGTTGTGGGGTATTTGGAAGAGGAGGCGAT 560
Y  F  L  T  Y  L  V  S  P  K  L  A  H  R  V  V  G  Y  L  E  E  E  A  I

CCACTCGTACACCGAGTTCTTGAAAGAGTTGGACAAGGGCACTATTGAGAATGTTCCTGCTCCGGCTATT 630
  H  S  Y  T  E  F  L  K  E  L  D  K  G  T  I  E  N  V  P  A  P  A  I

GCCATCGACTATTGGCGTATGCCACCAAACTCGACTCTGCGCGATGTAGTCATGGTAGTTAGAGCTGACG 700
 A  I  D  Y  W  R  M  P  P  N  S  T  L  R  D  V  V  M  V  V  R  A  D

AAGCTCACCACCGTGATGTTAACCATTTTGCATCGGACATTCATTATCAGGGACATGAACTGAAGGAATC 770
E  A  H  H  R  D  V  N  H  F  A  S  D  I  H  Y  Q  G  H  E  L  K  E  S

CCCAGCTCCACTTGGATATCACTGAATGGTCTGAAGAACTATACTGGAGAAGTATATTTAACCAATATCT 840
  P  A  P  L  G  Y  H  *

ATAATATCGAATAAGATATACTAATTTGTTAAATTTTCCTTTTCTTGAGATTGATTTCATTGTATGTTTT 910
TCACTGATCACATCATAATAAGTGTTGAATGTCTGTTATTTTACGTTTTTGGTTGAGAAATATCATGAAC 980
TAGTAATCGCTTTGCGTATTTATATTTCTTTTAAGGTATTTACTAAAAAAAAAAAAAAAA 1040
```

Fig. 9. Nucleotide sequence (*in silico*) and deduced amino acid sequences of cDNAs encoding *Olea europaea AOX1a, OeAOX1a*. * indicates the stop codon. For *in silico* construction of the *AOX1a* complete gene sequence was considered the largest 3'-UTR sequences (corresponding to clone 8 of Fig. 7)

Fig. 10. Nucleotide and deduced amino acid sequences of partial cDNAs (the sequence corresponds to a partial sequence of exon 3 considering the structure of four exons) encoding *O. europaea AOX1b*, *OeAOX1b* (Acc. No JX912720). * indicates the stop codon.

```
ACACCAAAATCAAAGCTCAAAATACAATTCAAATTATTTTGTGTTTATTTCTTTCTATATTCAAAATCCC 70
GAATTCCAATGAGCCAACGTACAATTTCTAGTATGGTTTTTCGACAGATGCAGTCGAATTTTTCATCTTT 140
        M  S  Q  R  T  I  S  S  M  V  F  R  Q  M  Q  S  N  F  S  S  F

TAGTAGTTCGATGAATAATGTCTCCAAGAACTACCGACCCGCAATCACTCATATTTTTGAGGCAAGGTAC 210
  S  S  S  M  N  N  V  S  K  N  Y  R  P  A  I  T  H  I  F  E  A  R  Y

TATAGTAGTAACCTAGGTTCAAAAGGTAACAAAGAAGACGAGCCAGCACAAGCTGTGAAATTCGATTCCA 280
 Y  S  S  N  L  G  S  K  G  N  K  E  D  E  P  A  Q  A  V  K  F  D  S

ACTTTGAAAACGTCGATGGCCAAAATGGTAAGGCCGTCGTAAGCAGCTACTGGGGAGTACCTCCGTCGAG 350
N  F  E  N  V  D  G  Q  N  G  K  A  V  V  S  S  Y  W  G  V  P  P  S  R

GGCGACCAAGGAGGATGGATCGCCCTGGCGATGGAATTGTTTTCGGCCATGGGAGACTTATAAAGCGGAC 420
  A  T  K  E  D  G  S  P  W  R  W  N  C  F  R  P  W  E  T  Y  K  A  D

ACTTCAATTGATGTGACAAAGCACCACAAGGCAACTACGTTCATGGACAAATTTGCCTATTGGACTGTTC 490
 T  S  I  D  V  T  K  H  H  K  A  T  T  F  M  D  K  F  A  Y  W  T  V

AATCTCTCAAATTCCCCACCTACTTGTTTTTTCAGAGACGCCACATGTGCCACGCTATGCTCCTAGAGAC 560
Q  S  L  K  F  P  T  Y  L  F  F  Q  R  R  H  M  C  H  A  M  L  L  E  T

GGTGGCAGCTGTCCCGGGCATGGTGGGGGGGATGCTCCTACACTTAAAGTCGATCCGGCGGTTTGAACAC 630
  V  A  A  V  P  G  M  V  G  G  M  L  L  H  L  K  S  I  R  R  F  E  H

AGCGGTGGTTGGATCAAAGCCCTTCTCGAGGAAGCGGAAAATGAGAGAATGCATCTAATGACATTCTTAG 700
 S  G  G  W  I  K  A  L  L  E  E  A  E  N  E  R  M  H  L  M  T  F  L

AACTATCCCAACCGAAATGGTACCAGAGAGCCCTAGTATTTGCTGTCCAAGGTGTATTTGCAAATGCATA 770
E  L  S  Q  P  K  W  Y  Q  R  A  L  V  F  A  V  Q  G  V  F  A  N  A  Y

CTTTGTGTCCTATGTTGTGTCCCCAAAACTTGCTCATCGCATAGTAGGCTACCTTGAAGAAGAGGCAGTG 840
  F  V  S  Y  V  V  S  P  K  L  A  H  R  I  V  G  Y  L  E  E  E  A  V

AATTCATACACTGAATTTCTAATTGATTTGGAGAAGGGCCTTGTTGAAAATAGACCGGCGCCGGCAATCG 910
 N  S  Y  T  E  F  L  I  D  L  E  K  G  L  V  E  N  R  P  A  P  A  I

CCATTGATTACTGGCAGTTGCCGTCGGAATCAACGTTAAAAGATGTTGTCACGGTTATCAGGGCAGACGA 980
A  I  D  Y  W  Q  L  P  S  E  S  T  L  K  D  V  V  T  V  I  R  A  D  E

GGCACACCATCGTGACCTTAACCACTTTGCATCGGTAAGAAAGATTTTCATTTTCCTTTTCGACGAAAAT 1050
  A  H  H  R  D  L  N  H  F  A  S  V  R  K  I  F  I  F  L  F  D  E  N

ATTCTTAATACTTCAAACTTGTTAGAAGAGGTAATTGATCAATATGGTACCAAAATCGAGAAAGTCTTTA 1120
 I  L  N  T  S  N  L  L  E  E  V  I  D  Q  Y  G  T  K  I  E  K  V  F

TAATAAAAATTAATAAATAATATTCTTCTACGTAGTAGTTTGAATTTGAGAAATGCTACACTTACAAAAT 1190
I  I  K  I  N  K  *

AGACCCTTGCCAGATCTTTCACATGTGCGGACCCCTACGTGGGACCGACCCCTACAATGAGGGGTCGATA 1260
AAACATCTGTAGGAAATTGTTACGTACACAGAGCAATTTCCTTTGAATTTTTGATGAAAGATCGCACAAT 1330
TCCTAGATATTTTGTCAAATAAGAAATTTTCGATAATAGATGACAATAAATGATATCTAGTAGTGGATAA 1400
ATAAAATAACTTATTTTTCTAATAATTAGGTCGTACAAAAATTTCTTTATTATTCAAGTGAATGTCTGGT 1470
CTAAGTACTAAAAGGGATTTTTGACACTATAATATATAAAAGTTCAATTTTTATTAATAATCTATAATTC 1540
GTTTAATGTCCTAATAAGGAGTAATAATTTGTGAAATGAAACAAAAAAAAAAAAAAA 1597
```

Fig. 11. Nucleotide (*in silico*) and deduced amino acid sequences of cDNAs encoding *O. europaea AOX1c*, *OeAOX1c* (Acc. No JX912721). * indicates the stop codon.

```
GCAGCGGTTCCGGGAATGGTTGGGGGGATGCTATTGCATCTCAGGTCTCTTCGCAAGTTCGAGCAGAGTG 70
 A  A  V  P  G  M  V  G  G  M  L  L  H  L  R  S  L  R  K  F  E  Q  S

GTGGATGGATTAAAGCATTACTTGAAGAAGCTGAGAATGAGAGGATGCACTTGATGACCATGGTAGAGCT 140
G  G  W  I  K  A  L  L  E  E  A  E  N  E  R  M  H  L  M  T  M  V  E  L

TGTCCAGCCTAAATGGTATGAGAGAGTGCTGGTGCTCACAGTTCAAGGAGTTTTCTTCAACGCTTTCTTC 210
  V  Q  P  K  W  Y  E  R  V  L  V  L  T  V  Q  G  V  F  F  N  A  F  F

GTGCTCTACTTGCTGTCTCCCAAACTGGCTCATAGAGTTGTTGGGTATTTAGAAGAGGAGGCAATACATT 280
 V  L  Y  L  L  S  P  K  L  A  H  R  V  V  G  Y  L  E  E  E  A  I  H

CATATACTGAATACTTGAAGGATATTGATAGTGGTAAAATCGAGAATGTCCCTGCTCCAGCTATCGCAAT 350
S  Y  T  E  Y  L  K  D  I  D  S  G  K  I  E  N  V  P  A  P  A  I  A  I

TGACTACTGGAGATTGCCTAAAGATGCAACTCTGAAGGATGTTATTACTGTTATCCGAGCCGACGAAGCA 420
  D  Y  W  R  L  P  K  D  A  T  L  K  D  V  I  T  V  I  R  A  D  E  A

CACCATCGTGATGTCAATCATTTTGCTTCTGATATTCATTTCCAGGGAAAGGAATTAAGGGAAGCACCAG 490
 H  H  R  D  V  N  H  F  A  S  D  I  H  F  Q  G  K  E  L  R  E  A  P

CTCCGGTTGGTTACCACTAGTTGTTGGTCGTGTGCTAGTTCATAATCGCGTTTTAATTGGTCCCGGTTTT 560
A  P  V  G  Y  H  *
GTTATACATTCTGAAAATCATCGTTGAAATAAAAATAGAGTCGGTGTCAAAAATTTATGGTTTATAATGA 630
TTCAATACTACAGAAACATGCCACTTACTTATGCCTGATCTGCCGCTAGTTTGGTCCATTCCCCGTCTAT 700
AGCTATTCTTAGTATAAGTTGAATTATGTTGTGTATCGTTATCCAAAATATTTTTGTAAGTTTGTTCTTT 770
GGTGGCTTAATATCCAATCAAATATGTAATCTCTTTAAAAAAAAAAAAAAA 821
```

Fig. 12. Nucleotide (*in silico*) and deduced amino acid sequences of partial cDNAs (the 5' end of the gene is not complete) encoding *O. europaea AOX*, *OeAOX2* (Acc. No JX912722). * indicates the stop codon.

OeAOX1a and OeAOX1c proteins location was predicted by using the MitoProt II 1.0 software. In case of OeAOX1c predicted length of mitochondrial transit peptide (Table 4) from the beginning of the protein to the cleavage site was 46 amino acids. However, in case of OeAOX1a no mitochondrial transit peptide was identified at the N-terminal protein site. This could be related with the presented short ORF, due to a short exon 1, also visible on the Fig. 13. Prediction of mitochondrial transit peptide for sequences used in the alignment of Fig. 13 shows no conservation across genes and species.

Table 4. Predicted cleavage site and length of the mitochondrial transit peptide in AOX1 proteins from different plant species.

Protein	Acc.	Protein size	Score	Cleavage site	Cleaved sequence
OeAOX1a	-	263	-	-	-
OeAOX1c	JX912721	353	0.9562	**46**	MSQRTISSMVFRQMQSNFSSFSSSMNNVSKNYRPAITH IFEARYY
AtAOX1a	D89875	354	0.8756	**63**	MMITRGGAKAAKSLLVAAGPRLFSTVRTVSSHEALSASH ILKPGVTSAWIWTRAPTIGGMRF
AtAOX1b	D89875	325	0.8453	**52**	MMMSRRYGAKLMETAVTHSHLLNPRVPLVTENIRVPA MGVVRVFSKMTFEK
AtAOX1c	AB003175	329	0.9932	**53**	MITTLLRRSLLDASKQATSINGILFHQLAPAKYFRVPAVG GLRDFSKMTFEK
AtAOX1d	AY072541	317	0.9923	**50**	MSYRSIYRTLRPVLSSSVQSSGLGIGGFRGHLISHLPNVRL LSSDTSSP
LeAOX1a	AY034148	358	0.9768	**71**	MMTRGATRMTRVVMGHMGPRYFSTTVLRNNPGTGV VGGVAAGLLHGLPANPSEKVAVTWVRHFSAMGSRS
LeAOX1b	AY034149	318	0.9506	**33**	MNRNAAMKISGLLMRQLRGEFLPRGGMVQIRH

Protein sequence alignment and phylogenetic tree were performed including genes of AOX1-subfamily since the analysis of OeAOX2 is well described in Chapter 3.

Sequence comparison of the complete protein-coding region confirmed the similarity in exon 2, exon 3 and 4, but identified clear differences in exon 1. Interestingly, OeAOX1c shows at the N-terminal protein site a very low similarity, in terms of length and sequence, compared to all the sequences of the alignment.

Conserved nucleotide positions near important functional sites, such as the conserved cysteine in position CysI and the di-iron-binding sites, common to AOX proteins, can discriminate between proteins belonging to AOX1 and AOX2-subfamylies (see Fig. 13). These sites have been recently highlighted by Costa et al. (2009) and Frederico et al. (2009b) (see arrows in Fig. 13). Both olive proteins (OeAOX1a and OeAOX1c) contain conserved cysteines, di-iron-binding sites and the currently highlighted conserved sites to distinguish AOX1 from AOX2 (Fig. 13). Notably, *Lycopersicon esculentum* AOX1b is exceptional in that it does not show a cysteine in CysI, but contains a serine (S) in this position. However, the same amino acid change was already reported in other species (Costa et al. 2009). It is also interestingly to notice that the protein sequences which belong to clade 2 in the dendrogram of Fig. 14

share common amino acids in specific position, for example the two amino acids upstream in the vicinity of CystI and one downstream.

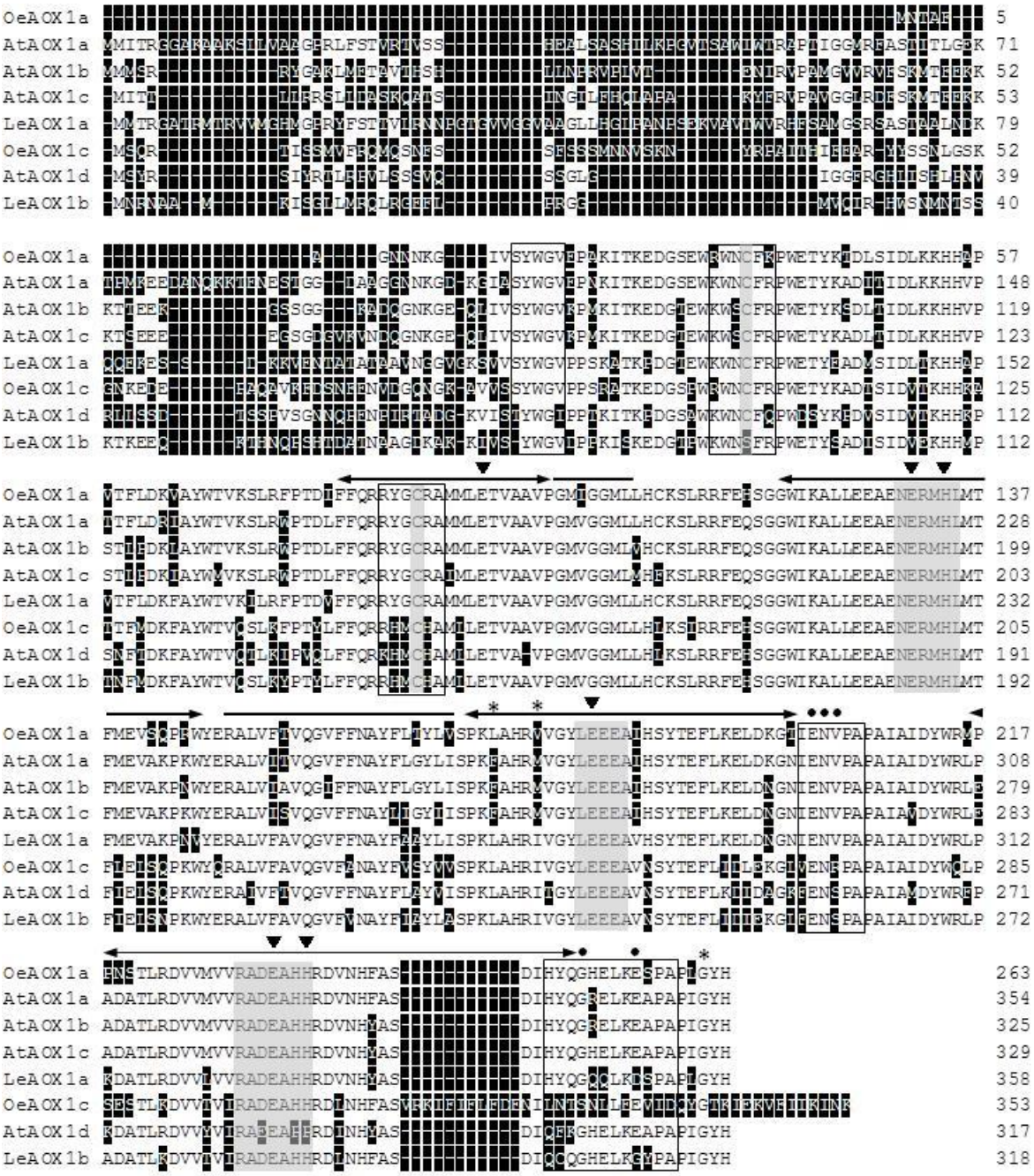

Fig. 13. Multiple alignment of the translated amino acid complete sequences of two *OeAOX1* (*OeAOX1a* and *OeAOX1c*) and 6 previously reported AOX1 from different plant species available at the NCBI. Amino acid residues differing are shown on a black background, deletions are shown by minus signs. The sites of two conserved cysteins (CysI and CysII) that are involved in dimerization of the AOX protein by S–S bond formation (Umbach and Siedow 1993) are indicated in grey boxes. In grey boxes are three regions defined by Berthold et al. (2000) as highly conserved in AOX. E (glutamate) and H (histidine) amino acids residues involved in the iron-binding are indicated by filled triangles. Asterisks indicate residues found to confer resistance to the AOX inhibitor salicylhydroxamic acid (SHAM) (Berthold 1998). At black boxes are the structural elements proposed to influence AOX regulatory behavior (Chrichton et al. 2005), the residues potentially involved in regulation of AOX activity are indicated by filled circles. Helical regions that are assumed to be involved in the formation of a hydroxo-bridged

binuclear iron center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown by two-headed arrows above the amino acid sequences. Possible membrane-binding domains center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown with line above the amino acid sequences. The peptide sequences presented in this figure refer to the ORF translation and the sequences given in figures 9 and 11. Accession numbers to published sequences are as follows: *OeAOX1a*, *OeAOX1c*, *AtAOX1a* (D89875), *AtAOX1b* (D89875), *AtAOX1c* (AB003175), *AtAOX1d* (AY072541), *LeAOX1a* (AY034148) and *LeAOX1b* (AY034149).

A phylogenetic tree was constructed by the Neighbor-Joining method with the AOX1 protein sequences also included in the alignment of Fig. 13, and one additional sequence used as out-group (AOX from the fungus *Neurospora crassa*) (Fig. 14). Both OeAOX1 sequences exhibit higher similarity with sequences of other plant species than between them. OeAOX1a belong to the bigger clade (Clade 1), while OeAOX1c belongs to a second clade composed with less sequences. It can be seen on clade 1, that OeAOX1a present low homology with all the other sequences, which could also be due to the shortness of the protein.

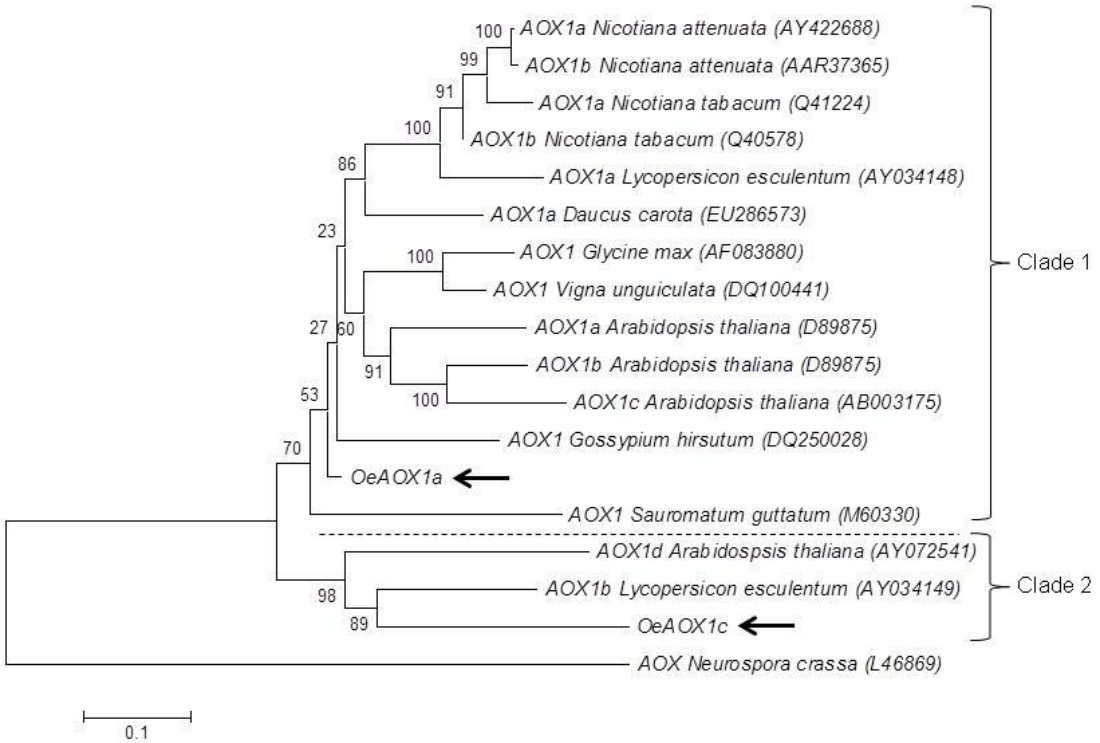

Fig 14. Dendrogram describing the relationship among AOX1 proteins from higher plants, including the two complete sequences of *O. europaea* (*OeAOX1a* and *OeAOX1c*). The alignment was bootstrapped with 1000 replicates by the Neigbor-Joining method using the MEGA 4 software. The fungus AOX *Neurospora crassa* was used as outgroup. The scale bar indicates the relative amount of change along branches.

2.5. Discussion

Olea europaea L. was shown to present genes from both *AOX*-subfamilies, AOX1 and AOX2. Thus, it follows a pattern which was expected for an eudicot plant species. Three *AOX1* genes (named *OeAOX1a*, *OeAOX1b* and *OeAOX1c*) and a single *AOX2* gene (*OeAOX2*) were identified and characterized. This finding places *O. europaea* L. into the most common group of plants, in which the *AOX* gene expansion occurs in the *AOX1*-subfamily. Phylogenetic studies based on amino acid sequence alignment showed that *OeAOX1* genes are more similar to genes from the same *AOX1* sub-family belonging to other plant species than to *OeAOX2*. In fact, *AOX1* and *AOX2* can be discriminated by conserved amino acid positions near important functional sites (Costa et al. 2009 and Frederico et al. 2009a). Nevertheless, the meaning of these sites and their conservation remain still obscure.

Sequence comparison of the complete protein-coding region confirmed the similarity to the corresponding sequence of exon 2 and exon 3 but identified clear differences in exon 1 sequence. A high similarity can also be observed among exon 4 sequences if *OeAOX1c* is not considered. *OeAOX1c* encodes a protein with a completely different C-terminus, not only in terms of the encoded peptide but also in terms of its length. This could represent a larger exon 4 size with a complete different sequence, or to the existence of an additional exon. Isolation of the complete gene at cDNA and gDNA levels will be still required to finally clarify the structure and sequence of the C-terminal region of the corresponding gene.

At the N-terminus, AOX presents much less similarity, which corresponds to the low homology among exon1. Campos et al. (2009) discussed the high variability in the predicted length of the mitochondrial targeting peptide located on that region. Finnegan et al. (1997) refer that lack of homology in mitochondrial targeting signals as common and typical for proteins that require N-terminal signals for mitochondrial import. Despite the length variability the mitochondrial targeting peptide should always be present since it is responsible for protein translocation to the mitochondria. The non-detection of mitochondrial targeting peptide in *OeAOX1a*, combined with the short protein length at N-terminus suggests that *OeAOX1a* is still incomplete at the 5'end. This suggestion could be reinforced by comparing the exon1 size across *AOXs*. It

is known that in *AOX* plant genes showing the typical four exon structure the last three exons have a conservation in size (exon 2: 129 bp, exon 3: 489 bp and exon 4: 57 bp) (Campos et al. 2009). Exon 1 is the only exon sequence that presents variation in its size, however, being normally higher than 300 bp (see details in Campos et al. 2009). Since both *OeAOX* genes present the four exons structure, we can hypothesize that exon 1 of *OeAOX1a* presents 114 bp and the exon 1 of *OeAOX1c* 384 bp. In fact, the exon size in case of *OeAOX1a* is very short which lead us to strongly consider that the previous suggestion might be correct. Nevertheless, further studies will be done to confirm the 5'end of that gene using a kit from a different brand. The FirstChoice RLM-RACE Kit (Ambion, Austin, TX, USA) was selected from the different brands available due to the guarantee of complete 5' ends isolation by using only complete mRNA strands for cDNA synthesis.

Differential regulation on both *AOX* gene subfamilies were described by several authors, and the differences on the functional sites identified between both subfamilies was pointed out as one reason. Besides differential regulation of the two AOX gene subfamilies may come from different positions in the plant genomes related to the chromosomal territories. For example, it can be observed that in *A. thaliana AOX1a*, *AOX1b* and *AOX1c* are located at chromosome 3, *AOX1d* at chromosome 1, whereas *AOX2* is situated at chromosome 5. Expression of the *AOX1* genes (such as tobacco and *A. thaliana AOX1a*) is highly responsive to abiotic and biotic stress, as well as dysfunctions in respiratory metabolism (Clifton et al. 2006). *AOX2* genes are generally not responsive, or at least much less responsive, to such conditions, being their expression more related with specific developmental and tissue expression (Saisho et al. 2001, Macherel et al. 2007, Chai et al. 2010). Nevertheless, several authors demonstrated also a role of *AOX2* in stress responses related to plastid signaling (Clifton et al. 2005) and to salt and drought stresses (Costa et al. 2007). Considering developmental plasticity upon stress that could result in AR, *OeAOX2* was primarily selected from the four *OeAOX* genes for the further initial studies.

Differences in gene regulation resulting in differences at the agronomic trait could be due to the existence of polymorphisms located in different regions of the gene body including the 3'UTR. Differential gene regulation by 3'UTR is mostly due to

the use of alternative polyadenylation (APA) sites, which could have influence at transcriptional or post transcriptional level. Selection of the incorrect poly(A) site could affect stability, translatability, and nuclear-to-cytoplasmic export (Zhao et al. 1999). Additionally, APA sites could also present a role at the control of messenger RNA (mRNA) metabolism and function by regulating the exclusion or inclusion of sequences which control the mRNA metabolism post-transcription (e.g. an miRNA binding site) (Xing and Li 2011). Several different reports show the existence of alternative polyadenylation (APA) in a variety of plant species and demonstrate its functionality in a range of biological process (see review in Xing and Li 2011). One of the best studied APA in plants involve oxidative stress response in *A. thaliana*, mainly from studies of the polyadenylation factor AtCPSF30 (Zhang et al. 2008), an ortholog of human CPSF30 (hCPSF30) and yeast Yth1p (yYth1p), both essential in cell viability and polyadenylation (Zhao et al. 1999). Additionally, several reports describe the involvement of APA on plant oxidative stress responses related to the NADPH oxidative *rbohA* in *A. thaliana* and chloroplast ascorbate peroxidase (*chlAPX*) in spinach and tobacco (Keller et al. 1998, Yoshimura et al. 2002). In olive, SNPs and single insertion/deletions (InDels) were identified in 3'UTR sequence of two *OeAOX* genes, the *OeAOX1a* and *OeAOX2*. Implications of those polymorphisms in AR efficiency can be investigated in future studies.

Transcription analysis of *OeAox1a* and *OeAOX2* revealed transcripts with variable length due to alternative polyadenylation. The production of mature transcripts with 3'-ends of variable length was already reported in plants (Shen et al. 2008). Xing and Li (2011) also reported that in *A. thaliana* 50% of genes have more than one unique poly(A) site, however, some of which may not be significant in terms of gene expression regulation. In *AOX* genes 3' ends variability was firstly reported in *AOX1* from maize (Polidoros et al. 2005) with a length range between 111-313 bp. Both *OeAOX1a* (length range between 160-245 bp corresponding to 203 bp in average) and *OeAOX2* (length range between 76-301 bp corresponding to 213 bp in average) tend to have similar sizes as demonstrated for *A. thaliana* and *Oryza sativa* transcripts showing 289 and 223 bp respectively in the average length of transcripts (Loke et al. 2005, Shen et al. 2008). Inclusion of key sequence elements for mRNA stability, location, or

suppression of gene translation could be related with *OeAOX* gene regulation. The *in silico* identification of a miRNA target binding site located in a polymorphic region of *OeAOX1a* indicate the possibility for gene regulation by posttranslational mechanisms. A miRNA target binding was also identified in the 3' end of the *OeAOX2* which is well described in chapter 3. The data obtained highlight potential mechanisms for *OeAOX1a* and *OeAOX2* regulation. Future experiments are required to elucidate the functionality of those putative miRNA target binding sites and its involvement in regulating of *AOX* gene expression.

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Chapter 3 Physiological responses and gene diversity indicate olive alternative oxidase (AOX) as a potential source for markers involved in efficient adventitious rooting induction

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3.1. Summary

Olive (*Olea europaea* L.) trees are mainly propagated by adventitious rooting of semi-hardwood cuttings. However, efficient commercial propagation of valuable olive tree cultivars or landraces by semi-hardwood cuttings can often be restricted by a low rooting capacity. We hypothesize that root induction is a plant cell reaction linked to oxidative stress and that activity of stress-induced alternative oxidase is importantly involved in adventitious rooting. To identify AOX as a source for potential functional marker sequences that may assist tree breeding, genetic variability has to be demonstrated that can affect gene regulation. The paper presents an applied, multidisciplinary research approach demonstrating first indications of an important relationship between AOX activity and differential adventitious rooting in semi-hardwood cuttings. Root induction in the easy-to-root Portuguese cultivar 'Cobrançosa' could be significantly reduced by treatment with SHAM an inhibitor of AOX activity. On the contrary, treatment with H_2O_2 or pyruvate, both known to induce AOX activity, increased the degree of rooting. Recently, identification of several *O. europaea* (Oe) *AOX* gene sequences has been reported from our group. Here we present for the first time partial sequences of *OeAOX2*. To search for polymorphisms inside of *OeAOX* genes, partial *OeAOX2* sequences from the cultivars 'Galega vulgar', 'Cobrançosa' and 'Picual' were cloned from genomic DNA and cDNA, including exon, intron and 3'-UTRs sequences. The data revealed polymorphic sites in several regions of *OeAOX2*. The 3'-UTR was the most important source for polymorphisms showing

5.7% of variability. Variability in the exon region accounted 3.4% and 2% in the introns. Further, analysis performed at the cDNA from micro shoots of 'Galega vulgar' revealed transcript length variation for the 3'-UTR of *OeAOX2* ranging between 76 and 301 bp. The identified polymorphisms and 3'UTR length variation can be explored in future studies for effects on gene regulation and a potential linkage to olive rooting phenotypes in view of marker-assisted plant selection.

3.2. Introduction

Olive propagation through efficient rooting of semi-hardwood shoot cuttings is of commercial interest. However, application of this methodology is limited to easy-to-root genotypes. Several valuable olive cultivars and breeding lines from diverse countries of the Mediterranean climate regions suffer from low rooting efficiencies at a rate around 20% or below. However, high variability in rooting phenotypes observed among olive cultivars and landraces point to the possibility for tree improvement in available olive material. Furthermore, rooting efficiency strongly depends on the physiological state of the tree or tree part, the environment and seasonal and annual changes.

Root induction at shoot cuttings originates from reprogramming of basal shoot cells. Shoot cutting and subsequent treatments with auxins constitutes a stress to the involved cells. Directed growth responses on stress are supposed to be a plant acclimation strategy to diminish stress exposure (Potter et al. 2007). Plant reactions upon stress involve hormone-transmitted metabolic changes, molecular transduction pathway activation, protein degradation and protein *de novo* synthesis as well as adaptive global genome regulation (see e.g. in Arnholdt-Schmitt 2004, Zavattieri et al. 2009). Restructuring of the inner and outer shapes of target cells, a change in the rate of growth and/or development and induction of adventitious organs, such as roots, shoots or hairs are visible signs of an acclimation strategy. Thus, stress acclimation is not physically possible without a change in energy allocation and spatial changes in metabolism (see discussion in Arnholdt-Schmitt et al. 2006a). Physiological and morphological plasticity are not occurring in a random way, but show components of

reproducibility that needs coordinated events at target cell levels, such as for example the formation of cluster roots or root hairs during phosphorous depletion stress (Shane et al. 2004) or of roots in olive shoots upon auxin treatment or the initiation of somatic embryogenesis by a reduction of the concentration of auxin in the medium (Frederico et al. 2009).

Treatment with exogenous auxin increases the number of meristematic root primordia. Meristem maintenance and development is known to be regulated by a balance of auxin and cytokinin (Loio et al. 2008). Both growth regulators are able to interact with reactive oxygen species, such as hydrogen peroxide (H_2O_2). H_2O_2 seems to play a crucial role in environmental stress perception and signaling related to the central role of mitochondria in homeostasis and cell fate determination under stress (Sung et al. 2005, Amirsadeghi et al. 2006). In guard cells auxin seems to limit H_2O_2 levels through preventing its generation, but unlike cytokinin it did not reduce exogenous H_2O_2 levels (Song Xi-Gui et al. 2006). Several reports are now available that point to a critical role of reactive oxygen species (ROS) signaling in root induction and development (Dunand et al. 2007, Nag et al. 2001). Seed germination of rice seedlings could be promoted by applying exogenous H_2O_2 (Sasaki et al. 2005). Treatment of olive shoot cuttings with H_2O_2 could increase early-rooting and the number of roots across genotypes and environments. However, the effects of the treatment differed clearly among olive cultivars and different years. Quantitative and qualitative differences in rooting between genotypes and varying environmental conditions during tree growth could not be equalized by H_2O_2 application. No interaction between years or cultivars and the treatment was obtained (Rugini et al. 1997, Sebastiani and Tognetti 2004). The strong influence of genetic factors on olive rooting efficiencies encourages searching for appropriate molecular markers for tree breeding.

Genes of interest for functional marker development can be identified by high-through-put differential gene analyses or in hypothesis-driven research approaches (Arnholdt-Schmitt 2005). Candidate gene approaches for marker-assisted selection are actually rated as the most promising strategies in molecular plant breeding (Collins et al. 2008). To verify a gene as functional marker candidate three prerequisites must be

fulfilled. The gene must be (1) importantly involved in the final trait, (2) the gene must show polymorphic sequences to mark genotypic differences and, finally, (3) the polymorphic marker must be stably linked to the target phenotype. The present paper deals with the first two aspects. The importance of a gene can be assessed by manipulating gene activities and physiological responses related to the target trait, through functional genomics, including transgenic strategies, linkage mapping and/or association studies. Once a gene is identified as candidate by a hypothesis-driven or an analytical approach, it is not necessarily needed to understand causal biochemical or molecular details of the gene function and complex regulation to develop a gene sequence as marker. Identification of a close relationship between the gene and/or the polymorphic sequence and the target trait is sufficient.

Respiration plays a central role in cell acclimation. Therefore, all genetic components directly involved in adaptive respiration can serve as promising candidates for functional marker development related to any stress reaction. The present paper focuses on the development of alternative oxidase (AOX) as a functional marker for adventitious rooting of olive shoot cuttings. AOX is nuclear encoded, but active in the alternative respiration pathway in mitochondria. It is positioned up-stream to important energy and carbon turnover regulation. AOX to cytochrome oxidase (COX) rate is an important measure for carbon efficiency rates related to environmental factors, growth and development (Hansen et al. 2009). The hypothesis that AOX can be critical for a stress reaction, such as adventitious rooting studied in this paper, was recently developed by Arnholdt-Schmitt et al. (2006a; see also Arnholdt-Schmitt et al. 2006b, Macedo et al. 2006a,b). This view got support through current knowledge on the central role of mitochondria and AOX in stress perception and cell signaling upon stress (Amirsadeghi et al. 2007, Vanlerberghe et al. 2009). AOX is known to be involved in plant reactions upon all types of abiotic and biotic stress. ROS seems to play a critical role for this link (Amirsadeghi et al. 2006). Several articles report about a complex interaction between AOX activity and H_2O_2 (e.g., Amirsadeghi et al. 2006, Gray et al. 2004, Popov et al. 1997, Umbach et al. 2005, Vanlerberghe and McIntosh 1996). Consequently, inhibitors of AOX activity, such as salicyl-hydroxamic acid (SHAM) were found to interact with H_2O_2 cell levels. In SHAM-treated *Arabidopsis*

thaliana roots, peroxidase was inhibited and root length was strongly reduced. H_2O_2 disappeared from the root hair zone, but was accumulated in cell walls in the meristem region (Dunand et al. 2007). H_2O_2 treatment of rice seedlings promoted germination and seedling growth and *AOX1a* was up-regulated by this treatment at an early stage (Sasaki et al. 2005). An up-regulation of *AOX* transcription upon H_2O_2-treatment was observed by Polidoros et al. (2005) in maize and maintenance of high levels of AOX activity was suggested to reinforce establishment also of maize seedling germination and growth under stressful environmental conditions (Camacho et al. 2004). Giraud et al. (2008) characterized an *aox1a* mutant of *A. thaliana* with reduced growth rates in early stages of seedling growth. Whereas *AOX1* subfamily genes are known to be related to diverse types of stress reactions, members of the *AOX2* gene subfamily are thought to have a closer relation to tissue specificity and development. However, this correlation is not fixed, since a relationship to stress-induction was also indicated for *AOX2* in *A. thaliana* and *Vigna unguiculata* (see the review Polidoros et al. 2009). Alternative respiration can be promoted through blocking the cyanine-sensitive respiration pathway via cytochrome oxidase. Potassium cyanide (KCN) treatments of dormant grapevine cuttings induced an increase of bud break and rooting (Mizutani et al. 1994), which can point besides of a role for COX also to the importance of AOX. *AOX* expression can be up-regulated in plant metabolism by organic acids, such as pyruvate (Juszczuk and Rychter 2003, Oliver et al. 2008). Therefore, it was used in our study besides H_2O_2 to test its effect as an external stimulator for olive root induction.

The importance of *AOX* genes in affecting physiological responses under determined conditions must be verified at species level since orthologous genes can have different functions in different species. Furthermore, the importance of a gene can be influenced by the genetic background of a defined genotype. Thus, a functional marker for rooting efficiency from *AOX* genes need to be developed at species level and with preference in genetic material that displays already the overall characteristics of a commercially important variety for breeding. A later introgression of additional genetic characteristics, such as quality traits of olive oil or other agronomic important tree traits will be possible under testing the stability of the potential functional marker for rooting efficiency. The present paper reports about AOX inhibitor and stimulator

studies with the easy-to-root cultivar 'Cobrançosa' and screening of a partial sequence of *OeAOX2* from cultivars 'Cobrançosa' and 'Picual', and the non-easy-to root cultivar or landrace 'Galega vulgar'. Here we publish for the first time initial experimental results that encourage further efforts to work on the hypothesis that alternative oxidase (AOX) gene sequences may serve as a source for functional markers for efficient adventitious rooting of olive shoot cuttings. However, the link between polymorphic AOX gene sequences, altered gene regulation and a rooting phenotype needs still to be approved.

3.3. Material and Methods

3.3.1. Rooting assays: treatment of shoot cuttings with IBA, SHAM, H_2O_2 and pyruvate

3.3.1.1. Plant material

Olive semi-hardwood shoot cuttings were obtained from 10-years old, field-grown mother plants of the easy-to-root Portuguese olive cultivar 'Cobrançosa'. Shoot cuttings with about 14 cm length were collected from the medium portion of one-year-old branches. The 'Cobrançosa' orchard has a polyclonal origin grown in a nursery company located in Montemor-o-Novo, Alentejo, in the southern part of Portugal. The orchard was established on a range of 1 m row and 3 m between rows. The plants had been trained as shrubs. Annually the orchard is submitted to a vigorous pruning to maximize the production of vegetative material for tree propagation.

3.3.1.2. Root assay conditions

The rooting trials were conducted in benches in a greenhouse cooled by *'aqua cooling'* and an automatic shading system. The benches were equipped with an intermittent sprinkling system and basal heating. Greenhouse temperature was maintained at 24ºC and the substrate temperature at 27 to 28 ºC. Shoots were cut to about 14 cm in length and only the top four leaves were maintained. After the treatments, the shoot cuttings were placed into the benches with '*perlite'* as rooting substrate.

3.3.1.3. Shoot cutting treatments

Shoot cuttings were treated with IBA (Sigma, Canada), which is used in commercial olive propagation as a root promoting auxin, the inhibitor of AOX activity SHAM (Aldrich, Germany) and two stimulators of AOX activity, H_2O_2 (J.T. Baker, Holland) and pyruvate (Fluka, Switzerland). All compounds were applied as water solutions through immersion of the cutting bases (4 to 6 cm). Due to their insolubility in water, IBA was previously soluted in a few drops of NaOH (Sodium hydroxide) 1N and SHAM in 95 % ethanol. For detailed information on used product concentrations and contact times with the cuttings bases, please refer to Table 1.

Table 1. Treatments of SHAM, H_2O_2 and Pyruvate applied to semi-hardwood olive shoot cuttings (for details see in Material and Methods).

Code	Treatment	Concentration [mM]	Contact Time
1	Negative control	-	-
2	IBA (positive control)	17	20 s
3	SHAM	10	20 s
4	IBA + SHAM	17 + 10	20 s
5	H_2O_2	10	1 h
6	H_2O_2	10	3 h
7	H_2O_2	10	6 h
8	H_2O_2	10	14 h
9	Pyruvate	0.01	3 h
10	Pyruvate	0.1	3 h
11	Pyruvate	0.01	20 s
12	Pyruvate	0.1	20 s

3.3.1.4. Experimental design and data analysis

The experimental conditions applied to SHAM trials correspond to a fully randomized scheme within a three-factorial design: 3 experimental periods x 4 treatments x 3 replications. Independent experiments were performed during March/April in 2007, October/November in 2007 and during May/June in 2008. Each replication with 20 semi-hardwood cuttings was used as an experimental unit for statistical data analysis.

The trials with hydrogen peroxide and pyruvate were also conducted applying a completely randomized scheme within a simple factorial assay with 6 treatments and 3 replications. For both trials, each replication with 10 semi-hardwood cuttings was used as experimental unit for statistical data analysis.

In all trials rooting rates were evaluated after 60 days and the collected data submitted to variance analysis using Statistic V.7.0 as software and the results are presented as 95% confidence interval charts.

3.3.2. Molecular studies - *OeAOX2* expression in root-inducible tissues of shoot cuttings

3.3.2.1. Plant Material

Shoot cuttings randomly collected from polyclonal 'Cobrançosa' trees were used for *OeAOX2* gene expression studies. The samples consist of olive tissue rings, each with about 1 cm high, taken from the basal portion of ten shoot cuttings as a bulked sample. The samples were collected in the field (T0), 12 h and 14 days after IBA treatment. Ten days after IBA application, root primordial induction can typically be observed by using histological techniques (Peixe et al. 2007a).

3.3.2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the bulked samples of 10 shoot cuttings and purified using the RNeasy plant mini Kit (Qiagen, Germany). Single strand cDNA was synthesized by help of the enzyme *RevertAid^{Tm} HMinus M-MuLV Reverse Transciptase* (Fermentas, Canada) using the oligo(dT) primer 5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV (V=A,C or G)-3' (Roche, Germany). The primer (2 µM) was incubated with 5 µg of total RNA for 5 min at 70 ºC. After this procedure the mixture was placed on ice for 5 min, and then the following reagents were added: 1x enzyme buffer *RevertAid^{Tm} HMinus M-MuLV Reverse Transcriptase* (Fermentas, Canada), 1 mg/ml of BSA (Ambion, USA), 1 mM each dNTP (Fermentas, Canada), 0,05 µg/µl of Actinomycin D (Roche), 2 mM of DTT (Fermentas, Canada), 0,8 U/µl of RNase inhibitor (Fermentas, Canada), and 6 U/µl of the enzyme *RevertAid^{Tm}*

HMinus M-MuLV Reverse Transcriptase (Fermentas, Canada). This reaction mixture was incubated for 90 min at 42 ºC.

3.3.2.3. Gene expression

RT-PCR was performed in final volumes of 25 µl with pure Taq Ready-to-go PCR Beads (GE Healthcare, England) using 1 µl of 1:10 cDNA dilution and 0.2 µM of each primer OeAOX2_1111Fw: 5'-CCACTAGTTGTTGGTCGTGTGC-3' and OeAOX2_1246Rev: 5'-AGTAAGTGGCATGTTTCTGTAG-3' (both from Eurofins MWG Operon, Germany). PCR was carried out for 35 cycles in the 2720 Termalcycler (Applied Biosystems, Singapore). Each cycle consisted of 30 s at 94 ºC for denaturation, 30 s at 55 ºC for primers annealing and 30 s at 72 ºC for DNA synthesis. An initial denaturation step at 94 ºC for 5 min and a final step at 72 ºC for 10 min were included. PCR products were analyzed by electrophoresis in 1.4% (w/v) agarose after staining in a EtBr solution (2 ng/ml) using the Gene Flash Bio Imaging system (Syngene, UK).

3.3.3. Gene diversity and 3'-UTR sequence and length variability

3.3.3.1. Plant material

To initiate studies on *OeAOX2* gene diversity at genomic DNA level, the DNA of young leaves from one tree of each of the three cultivars 'Galega vulgar' (clone 1053), 'Cobrançosa' and 'Picual' were isolated. The trees were grown in fields in Montemor-o-Novo ('Galega vulgar' and 'Cobrançosa') and Elvas ('Picual'), Alentejo, Portugal.

For cDNA studies, leaves of five micro shoots were extracted as a bulked sample. The micro shoots originated from an unknown number of trees of the clone 1053 of the cultivar Galega vulgar. The establishment of micro shoot cultures was performed as previously described by Peixe et al. (2007b).

3.3.3.2. DNA extraction

Genomic DNA (gDNA) was extracted using the DNEasy kit (Qiagen, Germany) according to the manufacturer's protocol. DNA integrity was assessed by electrophoresis in 1% agarose and DNA quantification was performed in agarose gels using defined amounts

of lambda DNA as a standard. Nucleic acids were visualized through EtBr staining as described before.

3.3.3.3. RNA extraction and cDNA synthesis

All steps were performed as described under II-2.

3.3.3.4. AOX2-specific fragment isolation from three cultivars

*AOX*2-specific primers were designed in the region of exon 3 (OAOX2_33Fw: 5'-ATTGCATCTCAGGTCTCTTCGC-3') and 3'-UTR (OAOX2_647Rev: 5'-CAGGCATAAGTAAGTGGCATG-3') (both from Eurofins MWG Operon, Germany) (see Fig. 1). For the PCR mix 0.02 U of a *Phusion™ High-Fidelity DNA Polymerase* (Finnzymes Oy, Finland) were used with 1x manufacturer supplied Phusion HF buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs (Fermentas, Canada) and 0.2 µM of each primer. The PCR was carried out with 0.2 ng/µl of gDNA as template running an initial step of 30 s at 98 ºC followed by 35 cycles, each consisting of 10 s at 98 ºC, 15 s at 58 ºC and 60 s at 72 ºC.

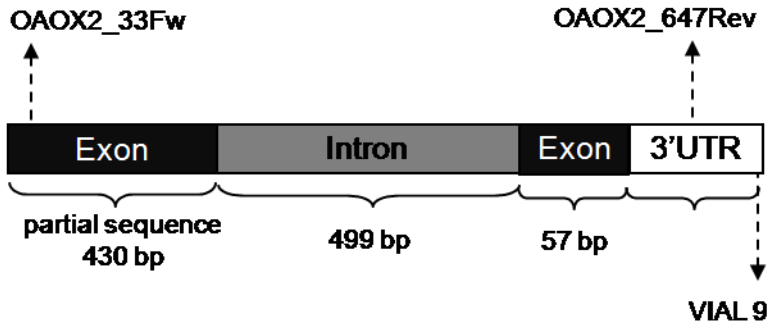

Fig. 1. Structure of the partial *OeAOX2* gene from *O. europaea* cv. 'Galega vulgar'.

PCR fragments were analyzed in 1.4% agarose gels and visualized as described. The single PCR fragment generated from each cultivar was purified using the GFX PCR DNA and Gel Purification kit (GE Healthcare, UK). The addition of adenines to the 3'end of amplicons required for the cloning procedure was made by adding 0.1 U/µl of *Taq* polymerase (Promega, USA), 1x manufacturer supplied $(NH_4)_2SO_4$ buffer, 2.5 mM

$MgCl_2$ and 0.2 mM dATP (Fermentas, Canada). The final mix was incubated during 30 min at 72 ºC in a 2720 Termalcycler (Applied Biosystems, Singapore). The amplicons were cloned into a pGEM®-T Easy System I vector (Promega, USA) and subsequently used for the transformation of *E. coli* competent cells JM109 (Promega, USA). Plasmid DNA of selected white clones was extracted using the alkaline lyses protocol (Sambrook et al.1989) and was characterized by the restriction enzyme *EcoR*I. Recombinant clones were used for commercial sequencing: 1 from 'Galega vulgar', 7 from 'Cobrançosa' and 4 from 'Picual'. All sequences resulted from at least two sequencing cycles of each plasmid DNA in sense and antisense directions, using specific primers located within the vector (T7 and SP6).

3.3.3.5. 3'-UTR characterization in cDNAs of 'Galega vulgar'

3'-UTR sequence and length variability was analyzed in transcripts of micro shoots (clone 1053). Amplicons were produced by the forward primer OAOX2_33Fw combined with the reverse primer Vial9: 5'-GACCACGCGTATCGATGTCGAC-3' (Roche, Germany).

The PCR reaction was performed with pure Taq Ready-to-go PCR Beads (GE Healthcare, England). Each reaction contained 10 ng sample DNA and ran at a concentration of 0.2 µM of each primer.

The PCR was carried out with an initial step of 5 min at 94 ºC followed by 35 cycles, each consisting in 30 s at 94 ºC, 90 s at 68 ºC and 10 min at 72 ºC. PCR fragments were analysed in 1.4% agarose gels, purified, cloned and selected as described above. Nine recombinants bacterial clones from 'Galega vulgar' provided from a PCR using DNA from a bulked sample of five micro shoots were used to study 3'-UTR variability.

3.3.3.6. Sequence analysis and sequence alignment

Selected clones were sequenced by commercial services through the MACROGEN company (www.macrogen.com) using the specific primers T7 and SP6 for the cloning vector (Promega). Sequence homologies in the NCBI data base (National Center for

Biotechnology Information, Be-thesda, MD) were studied by using the Blast algorithm (Altschul et al 1990) (http://www.ncbi.nlm.nih.gov) (BLASTX and BLASTN).

Genomic DNA and cDNA sequences were analyzed and compared using the programs EditSeq (Lasergene, GATC Biotech, Konstanz) and Bioedit (Hall 1999). For sequence alignment the Clustal W algorithm of Megalign (Lasergene, GATC Biotech, Konstanz) was applied.

The level of variability was calculated for each gene region (exon, intron and 3'-UTR) according to: nº of nts with variation*100)/size of the gene region.

For polymorphism analysis in the partial ORF of 'Galega vulgar', the available gDNA sequence was aligned with 9 cDNA sequences.

3.3.3.7. Computational prediction and validation of miRNA percursors and mature miRNAs in intron sequences

Putative miRNA precursors were searched in eight intron sequences of the *OeAOX2* gene from 'Galega vulgar', 'Cobrançosa' and 'Picual' by using the software miR-*abela*, which is publicly available at (http://www.mirz.unibas.ch/cgi/pred_miRNA_genes.cgi). For validation of potential pre-miRNAs the software MiPred was applied, which is publicly available at (http://www.bioinf.seu.edu.cn/miRNA/) (Jiang et al. 2008).

Prediction of the secondary structure of pre-miRNA was run on the web-based software MFOLD 3.1, which is available at (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) (Mathews et al. 1999, Zuker 2003). The criteria applied for screening the candidates of potential pre-miRNA were described by Xie et al. (2007). To screen the candidates of potential miRNAs the validated pre-miRNAs were run with the software miRBase::Sequences, which is publicly available at (http://microrna.sanger.ac.uk/sequences/search.shtml).

3.3.3.8. Computational prediction of target miRNA binding sites

To predict the existence of putative sites at the 3'-UTR for miRNA annealing the software miRanda v3.0 was applied by using the largest sequence from all samples (Gal_3).

3.4. Results

3.4.1. AOX inhibitor and activator effects on olive rooting

SHAM treatment of shoot cuttings

Figure 2 shows the rooting rates of shoot cuttings treated with SHAM for an average value of three independent experiments with 'Cobrancosa'. The positive control (code 2), which corresponds to the treatment with IBA, gave the highest rooting percentages, around 60% in average. SHAM treatment either alone (code 3) or in combination with IBA (code 4) significantly reduced the level of root induction to the values observed for the negative control. Rooting rates in the presence of SHAM reached maximal 28%.

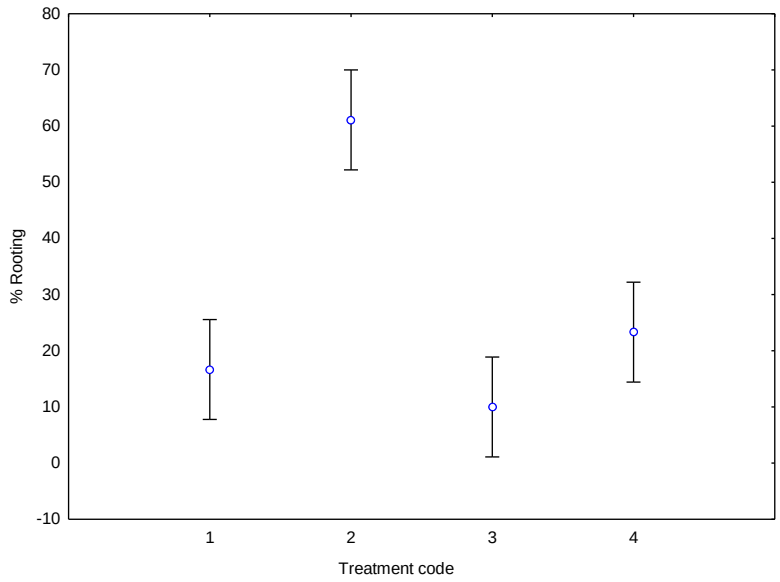

Fig. 2. Rooting rates observed in SHAM trial (LSD = 95%). (1: Control; 2: 17 mM IBA during 20 s; 3: 10 mM SHAM during 20 s; 4: 10 mM SHAM + 17 mM IBA during 20 s).

H_2O_2 treatment of shoot cuttings

The effect of H_2O_2 was time-dependent (Fig. 3). The 14 h treatment demonstrated a stimulating effect on rooting rates which achieved the same rooting rates as the IBA variant and, thus, being appropriate to substitute IBA treatments actually a common

practice in commercial olive propagation. A trend for increased rooting rates was recognizable from 3 h onwards, but it reached a degree near to statistical significance for the difference to the control only after a treatment for 14 h.

Pyruvate treatment of shoot cuttings

Pyruvate was applied at two different concentrations and contact times. A short pulse of 20 s (Fig. 4, treatment codes 11 and 12) was not sufficient to have any effect on rooting. This was observed independently from a concentration of 0.1 or 0.01 mM. However, when the time was prolonged to 3 h a stimulating effect was obtained that raised root rates to the level of the auxin-treated variant (Fig. 4, treatment code 10 in relation to 12). A similar stimulating effect through a longer contact time could be achieved by a 10 times lesser concentration (Fig. 4, treatment codes 9 and 10).

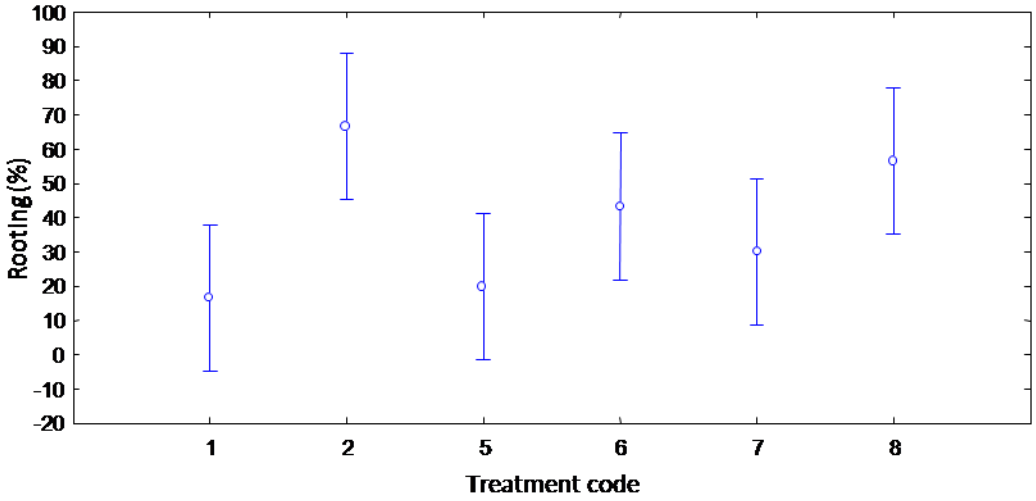

Fig. 3. Rooting rates observed in the H_2O_2 trial (LSD=95%). (1: Control; 2: 17 mM IBA during 20 s; 5: 10 mM H_2O_2 during 1 h; 6: 10 mM H_2O_2 during 3 h; 7: 10 mM H_2O_2 during 6 h; 8: 10 mM H_2O_2 during 14 h).

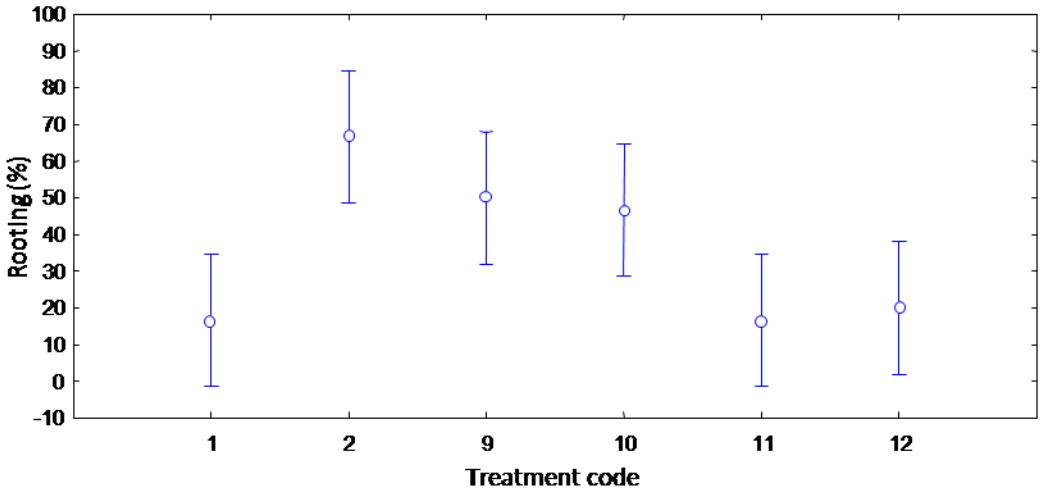

Fig. 4. Rooting rates observed in the Pyruvate trial (LSD=95%). (1: Control; 2: 17 mM IBA during 20 s; 9: 0.01 mM pyruvate during 3 h; 10: 0.1 mM pyruvate during 3; 11: 0.01 mM pyruvate during 20 s; 12: 0.1 mM pyruvate during 20 s).

3.4.2. *OeAox2* expression in shoot cuttings

Figure 1 shows the scheme of the partial *OeAOX2* sequence. The genomic sequence consists of a partial ORF of 475 bp, a complete intron of 499 bp and a partial 3'-UTR of 159 bp starting at the stop codon. The exon contains three of the four regions known as more conserved regions of the alternative oxidase (NERMHL, LEEEA and RADE__H region, Berthold et al. 2000), which include 5 di-iron binding sites (Fig. 5). Three helical regions that are assumed to be involved in the formation of a hydroxo-bridged binuclear iron center and a possible membrane-binding domain are included in that region (Siedow et al. 1995, Andersson and Nordlund 1999, Berthold et al. 2000).

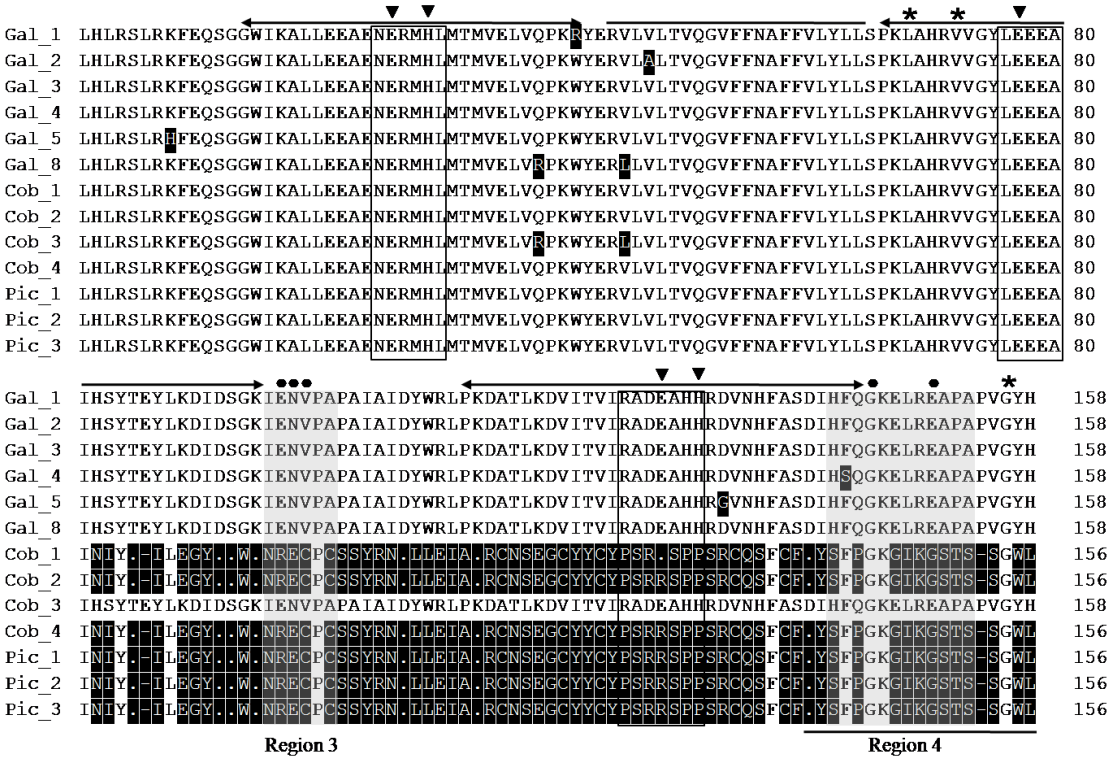

Fig. 5. Alignment of the translated amino acidic sequences obtained from the three genotypes of *O. europaea*. The underlined sequence corresponds to the peptide sequence of exon 4, all the other peptide sequences correspond to exon 3 in reference to *A. thaliana*. Amino acid residues differing are shown on a black background, deletions are shown by minus signs. Sites of translation stop are shown by points and are related with a *nonsense* mutation. In black boxes are three regions defined by Berthold et al. (2000) as highly conserved in AOX. The di-iron site positions are indicated by filled triangles. Asterisks indicate residues found to confer resistance to the AOX inhibitor salicylhydroxamic acid (Berthold 1998). In grey boxes are two structural elements proposed to influence AOX regulatory behavior (region 3 and 4 proposed by Crichton et al. 2005), the residues potentially involved in regulation of AOX activity are indicated by filled circles. Helical regions that are assumed to be involved in the formation of a hydroxo-bridged binuclear iron center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown by two-headed arrows above the amino acid sequences. Possible membrane-binding domains center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown with line above the amino acid sequences. The peptide sequences presented in this figure refer to the ORF translation and the sequences given in table 2.

Figure 6 demonstrates the expression pattern of *OeAOX2* during the root induction treatment in a bulked sample of shoot cuttings. Shortly after shoot cutting (10 min) still before the treatment with IBA, a high transcript level was observed that was later clearly reduced, as can be seen 12 h after IBA treatment. At the time, when histological evidence for root induction can typically be found (Peixe et al. 2007a), an increase in *OeAOX2* transcript level was obtained.

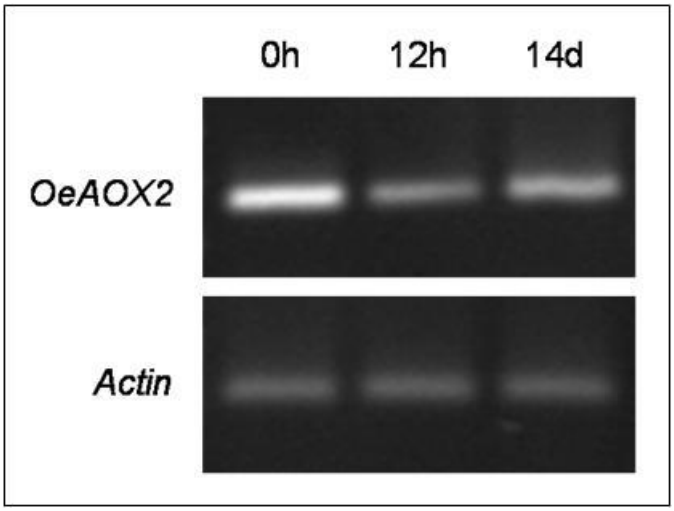

Fig. 6. Differential expression of *OeAOX2* in *O. europaea* cv. Cobrançosa at shoot cutting and during root induction in a bulked sample of 10 shoot cuttings (immersion during 20 s in a solution with 17 mM of IBA). RT-PCR was individually controlled by amplification of *Actin* mRNA.

3.4.3. Gene diversity revealed in *OeAOX2*

The partial sequence of *OeAOX2* was isolated from three cultivars ('Galega vulgar', 'Cobrançosa' and 'Picual') and DNA polymorphisms were identified. Table 2 presents the DNA polymorphisms that were identified among all available sequences.

Open reading frame (ORF)

Out of 10 ORF sequences identified from 'Galega vulgar' (9 provided from cDNA and 1 from gDNA), 6 presented variations between each other (Table 2). 12 SNPs were detected among the sequences of this cultivar in the conserved region of exon 3 and one SNP in the exon 4 region. From these 13 SNPs, 7 were responsible for non-synonymous translations: 23C/A→K/H; 114A/G→Q/R; 122T/C→W/R; 134G/C→V/L; 141T/C→V/A; 399A/G→D/G; 429T/C→F/S (Fig. 5). In sequence 4 a substitution of Phe (F) by Ser (S) due to the SNP at position 429 occurred within one of the structural

elements in the fourth helical region proposed to influence AOX regulation (Siedow et al. 1995, Andersson and Nordlund 1999, Crichton et al. 2005). In sequence 5 the Asp (D) / Gly (G) substitution due to a SNP in position 399 occurred two positions upstream the di-iron binding site of RADE_ _H box, which is included in the fourth helical region, previously assumed to be involved in the formation of a hydroxo-bridged binuclear iron center (Siedow et al. 1995, Andersson and Nordlund 1999). A multiple alignment made with the peptide sequences of plant *AOXs* available at the NCBI data basis showed at this position an Asp in all sequences (data not showed). In sequence 8 a Gln (Q) /Arg (R) substitution by a SNP in position 114 was identified eight positions upstream of the di-iron binding NERMHL box, included in the second helical region (Siedow et al. 1995, Andersson and Nordlund 1999). Variation at the possible membrane binding domain were observed in the sequences 8 and 2 due to a Val (V) / Leu (L) (SNP at the position 134) and a Val (V) / Ala (A) (SNP at the position 141) substitution, respectively.

TABELA 2

From 7 gDNA sequences identified as *OeAOX2* from 'Cobrançosa', 4 presented variations (Table 2). Among these sequences 6 SNPs were identified. All SNPs are repetitive, since they were already observed in 'Galega vulgar'. Additionally, an insertion / deletion (InDel) of two nucleotides (nts) was identified at positions 248 and 249. From these 8 sources of variation, 6 were responsible for synonymous translations and 2 SNPs were responsible for non-synonymous translations (114A/G→Q/R; 134G/C→V/L). In sequence 3 a Gln (Q) / Arg (R) substitution due by the SNP at position 114 (A/G) was observed at the second helical region (Siedow et al. 1995, Andersson and Nordlund 1999). Sequence 3 presented a mutation due to Val (V) / Leu (L) substitution (SNP at the position 134), which is included in the possible membrane binding domain (Andersson and Nordlund 1999, Berthold et al. 2000). The InDel converts the codon ACT to a premature stop codon (TGA) at nucleotide 255 of the coding region, named *nonsense* mutation. This mutation was detected in 5 of the 7 sequences from this cultivar. Two sequences of the 7 were identical to sequence 2 in Table 2. The stop codon is located 160 bp upstream of the 3'-most exon-exon junction and five positions upstream the di-iron binding site of the LEEEA box (Fig. 5).
Only 2 partial *OeAOX2* sequences from 'Cobrançosa' can be translated to a peptide (only sequence 3 is presented in Table 2) (see Cob_3 in Fig. 5).

Four gDNA sequences were cloned from 'Picual' and 3 of them varied between each other (Table 2). However, the ORF sequences are identical, and all presented the same *nonsense* mutation that was described before in 'Cobrançosa' (Fig. 5). All SNPs identified in 'Picual' were repetitive, i.e. the same variation has been identified already in at least one of the other two cultivars (Table 2).

In summary, the ORF of *OeAOX2* displays a rich source of polymorphic sites of which most are found to be repetitive within sequences from one cultivar and between cultivars. However, 'Galega vulgar' is the only one, which showed unique SNPs that could only be discovered in this cultivar (positions 23, 25, 122, 141, 196, 370, 399 and 429). No non-sense mutation was observed in the 10 sequences of this cultivar as described for both other cultivars. However, it must be considered that 9 of the sequences were deduced from actively transcribed sequences (cDNA) (Table 2).

Intron

All intron sequences from the three cultivars show the typical nuclear DNA exon-intron junctures 5'-GT/AG-3' (Saisho et al. 1997) (Table 2). The intron region is more U-rich than the flanking exons (41% vs. 28% U on average). Similar results were previously reported in *A. thaliana* showing 41% U vs. 26% U on average for introns (Goodall and Filipowicz 1989, Ko et al. 1998, Deutsch and Long 1999). It was suggested that U-richness can be important for intron recognition (Ko et al. 1998).

The intron of 'Cobrançosa' presented 7 SNPs and 1 InDel among the four sequences (Table 2). In the intron of 'Picual', 5 SNPs and 1 InDel have been identified. All SNPs identified in the intron are repetitive between cultivars, which means they have not been single events that occurred only in one cultivar, but could be observed in at least two cultivars. In all variable positions no more than two different nucleotides can be found. This was also observed in the exon.

In all introns from the three cultivars a putative miRNA precursor could be predicted (Table 3). However, after computational validation using the software MiPred, only 2 sequences of 'Cobrançosa' (1 and 3) presented a potential miRNA precursor with a higher probability of 65.9% (P-value=0.004 considering a shuffle time of 1000) (Table 3). Analysis of the predicted region allows the identification of variability between these two sequences of 'Cobrançosa' and all the other sequences analyzed. Two substitutions in the sequences Cob_1 and Cob_3 were identified: a substitution of C by G (position 703) combined with the substitution of G by T (position 754) (see Table 2).

Table 3. Computational prediction of intronic miRNA precursors in *OeAOX2* of three *Olea europaea* cultivars: 'Galega vulgar' (Gal), 'Cobrançosa' (Cob) and 'Picual' (Pic). *bp*: length of the pre-miRNA sequence in bp, *MFE*: minimal free energy in kcal/mol, *Prob*: probability to be a real pre-miRNA sequence.

Genotype	Putative pre-miRNA sequence	bp	MFE	Prob.
Gal8	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUU UGCCUGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	82	-13.22	no
Cob1	UUUUUUGUUGUCGAAAAUAGGAAUUUGUUUGAUUUCAAUUA AUAAAUAGUCAUUCUGUUUAGAUAAUAAGAAA	73	-16.30	65.9%
Cob2	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUU GCCUGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	-11.72	no
Cob3	AUAGUAUUUUGUCAUUUUUUUUGUCCCCUAUUAUCCACCCUUU UGCCUGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	82	-13.12	no
	UUUUUUGUUGUCGAAAAUAGGAAUUUGUUUGAUUUCAAUUA AUAAAUAGUCAUUCUGUUUAGAUAAUAAGAAA	73	-16.30	65.9%
Cob4	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUU GCCUGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	-11.72	no
Pic1	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUU GCCUGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	-11.72	no
Pic2	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUU GCCUGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	-11.72	no
Pic3	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUU GCCUGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	-11.72	no

Micro RNA precursors possess a characteristic secondary structure, with a terminal loop and a long stem (Bartel 2004) by which the miRNA is positioned (Reinhart et al. 2002). The secondary structure of the predicted pre-miRNA is shown at the figure 7.

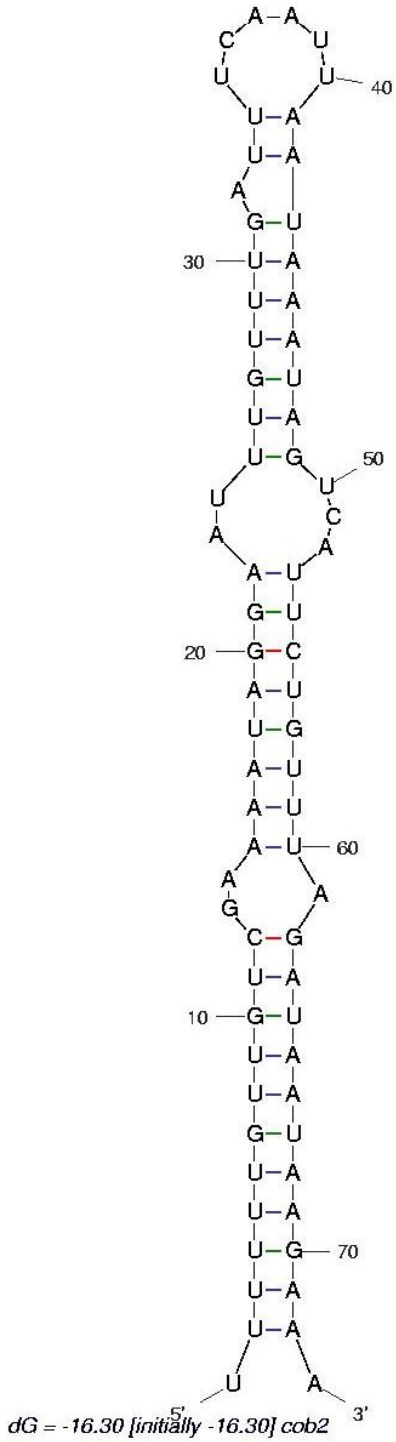

Fig. 7. Secondary structure of miRNA precursor predicted in the *OeAOX2* intron sequence of *O. europaea* cv. Cobrançosa.

The sequence between 13 and 32 nt (5'-GAAAAUAGGAAUUUGUUUGA-3') was identified with homology to a miRNA of *A. thaliana* ath-miR417 (78-GAAGGUAGUGAAUUUGUUCGA-98) with a score of 91.7. The ath-miR417 had been validated by Northern blot hybridization (Wang et al. 2004). Additionally, the sequence between 13 and 29 nt was extracted with homology to a miRNA of *Oryza sativa* osa-miR417 (44-GAAUGUAGUGAAUUUGUUCCA-64) with a score of 82.5 (see Fig. 8).

Fig. 8. Identification of a putative miRNA in *O. europaea*. Alignment of the predicted pre-miRNA of *OeAOX2* with ath-miR417 and osa-miR417.

3' Untranslated Region (3'-UTR)

Among the 3'-UTRs of 'Galega vulgar' sequences 5 SNPs and two single nt InDels were identified (Table 2). In the cultivar 'Cobrançosa' this region demonstrated 5 SNPs and two single nt InDel. In 'Picual' the 3'-UTRs of sequence 1 and 3 are identical, however, between these and sequence 2 4 SNPs and a single nt InDel were detected. Whereas most SNPs were repetitive between the cultivars, 'Galega vulgar' showed a unique SNP in position 996 and a deletion in position 1063. Cultivar 'Cobrançosa' demonstrated a unique deletion in position 1058.

Comparing the three regions within the partial genomic gene sequence as sources of polymorphisms among all studied sequences of the three cultivars, the ORFs presented 16 sites of variation which means a variability of 3.4%, the introns 10 sites equal to 2% and the 3'UTRs 9 sites which means 5.7% of variability. Calculations were based on the known sequence of each region (475 bp of ORF, 499 bp of intron and 159 bp of 3'-UTR).

The available sequence data for individual trees are restricted by the low number of studied bacterial clones (7 bacterial clones analyzed in cv. Cobrançosa and 4 in cv. Picual. For 'Galega vulgar' only one sequence was available). However, the data for the tree of 'Cobrançosa' and 'Picual' reveal each the existence of heterogeneity for *OeAOX*2 through four different *OeAOX*2 sequences in 'Cobrançosa' and three varying sequences for 'Picual'. In both cultivars, the number of polymorphic gene sequences was enhanced by considering the intron level: 'Cobrançosa' showed three different sequences in the ORF and also three different sequences in the 3'UTR, however, four different sequences were identified in the intron. 'Picual' demonstrated one identical sequence for the ORF, but two different sequences in the 3'UTR, and three polymorphic sequences considering the intron. Olive can be expected to be diploid. However, since at this stage no genetic analyses were performed in this study, the results should be seen as varying sequences, but can not be interpreted as allelic variation.

3.4.4. 3'-UTR sequence and length variability in transcripts of *OeAOX2*

Transcript analyses of the *OeAOX2* were performed from a bulked sample of five micro shoots from cv. Galega vulgar. The data obtained highlight potential mechanisms for *OeAOX2* regulation and were included for demonstration. Table 4 shows the length of the 3'-UTR and the number of clones detected for each 3'-UTR size in 'Galega vulgar'. Transcripts of variable length resulted from alternative polyadenylation (AP). AP affects a large number of higher eukaryote mRNAs, producing mature transcripts with 3'-ends of variable length.

Table 4. Alternative polyadenylation of the *OeAOX2* transcripts ('Galega vulgar') detected by 3'-RACE experiments. The length of the 3'-UTR in bases, the number and percentage of clones detected for each 3'-UTR size and the nomenclature of the sequence are shown.

3'-UTR size (bp)	Number of clones	Sequence identification
76	1	Gal_1
113	1	Gal_6
151	1	Gal_5
259	1	Gal_7
297	1	Gal_2
298	2	Gal_3
301	2	Gal_4

Alternative polyadenylation is an important mechanism in generating a diversity of mature transcripts. Conventional genetic mutagenesis studies revealed that plant poly(A) signals are composed of three major groups: far upstream elements (FUE), near upstream elements (NUE; an AAUAAA like element characteristic in animals), and cleavage elements (CE) (Loke et al. 2005). The composition of plant consensus signals, such as CEs, which is an expansion of the CS (cleavage site), including the YA dinucelotide (CA or UA) in the CS and two U-rich regions, one before and another after the CS, both spanning about 5 to 10 nt (Loke et al. 2005). NUE is an A-rich region and spans about 6 to 10 nt located between 13 and 30 nt upstream of the CS (referred to as locations -13 to -30; Hunt 1994, Li and Hunt 1995). FUE, the control or enhancing element, is a combination of rather ambiguous UG motifs and/or the sequence UUGUAA (Hunt 1994) and spans a region of 25 to 125 nt upstream of the NUE (Loke et al. 2005). The alternative sites for the poly(A) signal are indicated in figure 9.

In search for these nuclear mRNA poly(A) signals different regions of FUE and NUE elements were detected (Fig. 9). All identified FUE regions in olive *OeAOX*2 include different patterns found previously in *A. thaliana* (Loke et al. 2005) as the top 50 FUE patterns: the first region in *OeAOX*2 named FUE.1 (Fig. 9) include the pattern UUGUUG found as 25th most frequent pattern in *A. thaliana*; the FUE.2 GUUUUGUU include the patterns UUUGUU (1st), UUUUGU (2nd) and GUUUUG (22th); the FUE.3

region UGUUGUGU are related to the 21th and 23th patterns identified in *A. thaliana* (UGUUGU and UUGUGU, respectively); the FUE.4 region, UAUUUUUGUAAGUUUGUUCUUU, is the largest region and belong to a group of seven patterns previously identified in *A. thaliana*: 2nd, 7th (UUUUUG), 9th (UUCUUU), 14th (UUUGUA), 16th (AUUUUU), 18th (UAUUUU) and 47th UUGUAA). The pattern 9th in *A. thaliana* had been identified as the 6th most frequent in *O. sativa* (Shen et al. 2008). This last region include also the pattern UUGUAA referred by Hunt (1994) as typical for the FUE element.

NUE elements are A-rich and three were identified in *OeAOX*2. NUE.1 is common to the second NUE pattern (UUAAUU) identified in *O. sativa* (Shen et al. 2008); NUE.2 corresponds to the pattern AAUAAA, highly conserved in vertebrate cells, and NUE.3 refers to AAAUAU. Gal_2 demonstrates a deletion in NUE.2.

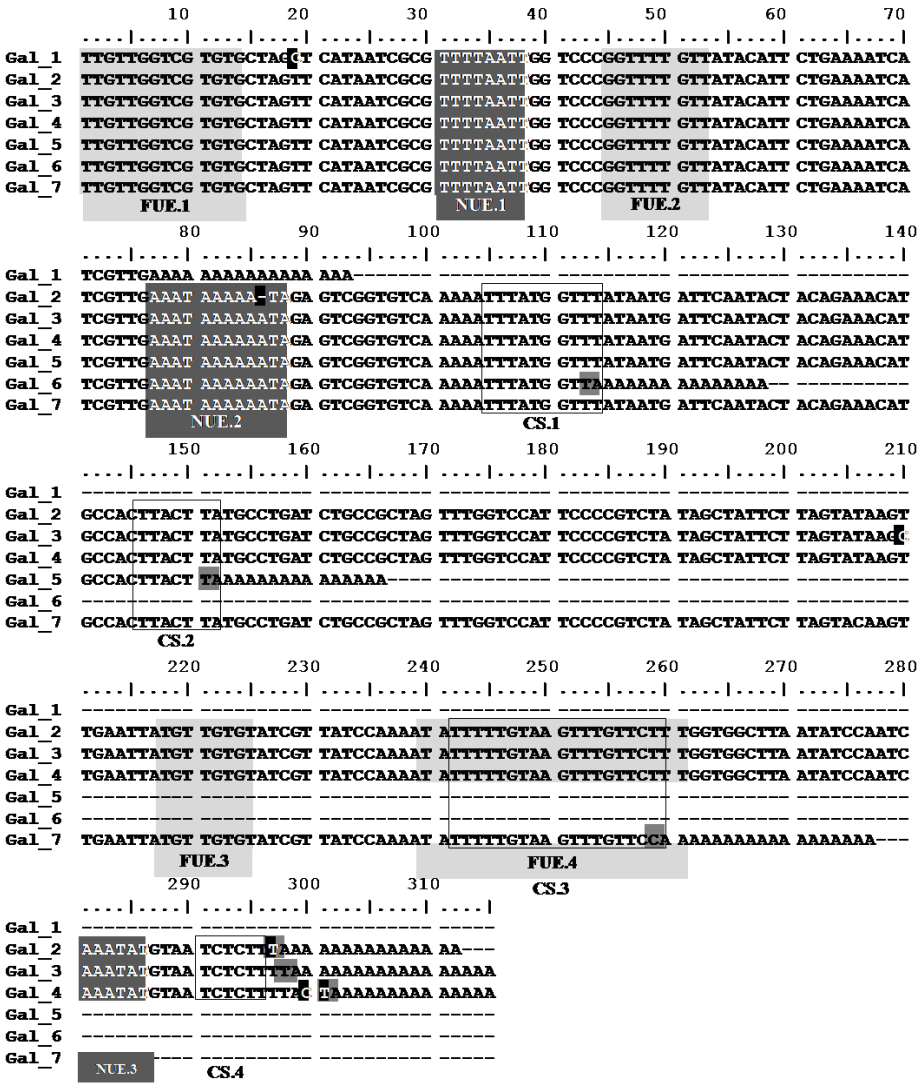

Fig. 9. Alignment of the 3'-UTR sequences identified in seven different *OeAOX2* transcripts of *O. europaea* cv. Galega vulgar.

The four FUE regions identified are complete and can be suggested to be functional for the NUEs downstream of each one. Gal_1 contains the shortest 3'-UTRs and ends at the beginning of NUE.2 in the other sequences. No CS element was discovered. For all other sequences, Gal_2 to Gal_7, a CS element could be identified, which included the di-nucleotide YA corresponding to TA or CA. The di-nucleotide is in different positions in the 3'-UTRs: for Gal_6 in position 113, Gal_5 in 151, in Gal_7 in 259, Gal_2 in 297, Gal_3 in 298, and for Gal_4 in position 301. In all sequences a T-rich region was observed upstream the CS element that is characteristic for the cleavage element.

Table 5 shows predicted target sites for miRNAs in the 3'-UTR of Gal_3. Many miRNA families are evolutionary conserved across all major lineages of plants, including mosses, gymnosperms, monocots and dicots (Axtell and Bartel 2005). This conservation makes it possible to identify homologous miRNAs known from other species (Floyd and Bowman 2004, Axtell and Bartel 2005). A previous study has shown that most known plant miRNAs bind to their mRNA targets with perfect or nearly perfect sequence complementarity and degrade the target mRNA. The plant miRNAs targets sites are mostly located in the protein-coding sequence acting in a way similar to RNA interference (Wang et al. 2004), but can be found also in the 3'-untranslated region (Rhoades et al. 2002). Five putative target binding sites for miRNAs could be predicted within regions of the 3'-UTR in Gal_3 sequence (Table 5). The only information available about this binding sites is related to the ppt-miR1212 localized in the 3'-UTR of *A.thaliana* B-box zinc finger coding-protein sequence (Talmor-Neiman et al. 2006).

Table 5. miRNA target sites predicted at the 3'-UTR region of the sequence Gal_3 (software miRanda v3.0).

miRNA identification	nt position	Alignment	Energy (kCal/Mol)
ath-miR401 (Sunkar and Zhu 2004)	2-23	Query: 3' acAGCCAGC-UGUGGUCAAagc 5' |:||||| :::|:|||| Ref: 5' tgTTGGTCGTGTGCTAGTTcat 3'	-22.10
	35-51	Query: 3' acAGCCAGCUGUGGUCAAAGc 5' |:|||| ||:|||||: Ref: 5' aaTTGGTC----CCGGTTTTg 3'	-20.10
ppt-miR1212 (Talmor-Nieman et al. 2006)	27-47	Query: 3' gcGUAAGAUACGACAGGGUgc 5' |:||:|| : |||||: Ref: 5' cgCGTTTTAATTGGTCCCGgt 3'	-21.36
smo-miR1110 (Axtell et al. 2007)	153-171	Query: 3' agGAACUGGUGACGGGGAUCg 5' | |||: ||||| |||| Ref: 5' tgCCTGAT--CTGCCGCTAGt 3'	-22.56
tae-miR1125	158-177	Query: 3' ggcGGCGUCAACCAGAGCAACCAa 5' |:||		|| ||||| Ref: 5' gatCTGCCGCTAGT----TTGGTc 3'	-23.97

3.5. Discussion

The presented research focuses on application. It is the aim of these studies to validate 1) the involvement of *AOX* genes in root induction of olive shoot cuttings and 2) to reveal whether *AOX* genes can be a source of polymorphic sequences that may be developed in a later step as functional markers for the selection of individual trees with a more efficient root induction. Thus, it is not the primary goal of this research to contribute to fundamental knowledge in AOX research, i.e. to understand why and how AOX is involved in the root induction process.

We applied the inhibitor of AOX activity, SHAM, and two compounds, H_2O_2 and pyruvate that are known to stimulate AOX activity (Feng et al. 2008, Oliver et al. 2008). SHAM is known to inhibit the alternative pathway. It was not the aim of this study to quantify electron partitioning between both respiration-pathways (Lambers et al. 2005), but to observe the effect on a physiological response. The results confirmed our expectation. SHAM strongly reduced root induction in a reproducible manner and both stimulators tended to increase rooting. Additionally, the results seem to confirm that root induction is an example of a stress-related cell reprogramming event as hypothesized (Arnholdt-Schmitt et al. 2006a, b). *OeAOX2* was highly expressed 10 min after shoot cutting before the IBA treatment, decrease rapidly and was then increased at the time, when root primordia started to be visible at histological level (Peixe et al. 2007a). Further, application of H_2O_2 as an important component of oxidative stress signaling succeeded to increase rooting. The current state of knowledge on an interaction of ROS and AOX suggests that AOX plays an important role in preventing ROS production, but also in the perception and coordination of oxidative stress signaling that influence mitochondrial dysfunction, the mitochondrial retrograde signaling pathways and cell recovery or cell death strategies (Fiorani et al. 2005, Umbach et al. 2005, Amirsadeghi et al. 2006, Clifton et al. 2006, Giraud et al. 2008, Van Aken et al. 2009). The experiments presented indicate an involvement of both AOX and H_2O_2 in olive rooting under the applied conditions used for commercial

application. The results are sufficient encouragement for our applied research strategy to go ahead and search for polymorphisms in *AOX* genes that can be related to differential gene regulation and subsequently for the rooting process. This strategy is bearing in mind that it would be already sufficient for marker-assisted breeding to find a close association or correlation between a polymorphic sequence related to *AOX* and the efficiency of the rooting process.

The presented studies on polymorphic *AOX* sequences are at this stage a first approximation and restricted to the identified *OeAOX2* gene in search for variability. The partial sequence from three cultivars contains part of the ORF, one intron and the 3'-UTR regions. Recent knowledge on *AOX* gene regulation suggests that differential regulation cannot be sufficiently explained by defined motifs in the promoter region (see review Polidoros et al. 2009). Variation in within-gene sequences at genome level and 3'-UTR micro-heterogeneity are currently considered as important factors that might cause diseases and differential regulation in genes (Goto et al. 2001, Lambert et al. 2003, Novelli et al. 2007). The principle aim of this study was to highlight the existence or non-existence of polymorphic sequences within the selected *OeAOX*2 gene as an example.

Several SNPs and InDels revealed *OeAOX2* as a rich source for polymorphic sequences. Sequence differences were identified from all gene regions. Highest variability was discovered for the 3'-UTR region, followed by the ORF and the intron. The relatively low variability in the intron is surprising, since introns are typically known to exhibit a higher variability than protein-coding sequences (Gibbs 2003). Since most parts of the ORF consist of the highly conserved region related to exon 3 from *A. thaliana* (Saisho et al. 1997), this was expected to be especially true. Interestingly, the majority of the SNPs identified in a sequence of one cultivar were found again in sequences from other genotypes. However, eight SNPs could be identified in the ORF of 'Galega vulgar', which could be observed only in this cultivar. Another unique SNP was found in the 3'-UTR of the same cultivar. This is of special interest for our approach, since this cultivar is a bad rooting cultivar and we are looking for differences that can be explored in future studies for any relationship to the rooting ability. Abe et al. (2002) reported the existence of a SNP in the *AOX1a* of *O. sativa* (*OsAOX1a*) leading

to a non-synonymous translations, that was completely linked to the presence of the QTL for low temperature tolerance. Five of the eight SNPs in the ORF of 'Galega vulgar' were responsible for non-synonymous translations. The substitution of Asp (D) by Gly (G) in position 399 in a sequence of 'Galega vulgar' (Table 4) indicates a position near the di-iron binding site of RADE_ _H box where Asp is found to be highly conserved among diverse species. The effect of substitutions in neighbour residues of the di-iron binding sites had already been demonstrated. Nakamura et al. (2005) reported in *Trypanosoma vivax* 70% reduction of the enzyme activity by artificial site-direct mutations in the conserved Glu to Ala positioned one and two positions downstream the L*E*EEA box di-iron binding site proposed by Berthold et al. (2000). Albury et al. (2002) performed the same site-direct mutation two residues downstream the di-iron binding site by changing the conserved residue to Asn and achieved total inactivation of the enzyme in *Sauromatum guttatum*.

The *OeAOX2* sequence of 'Cobrançosa' and 'Picual' carried an InDel mutation of two nucleotides (at positions 248 and 249) in exon 3 (considering the most conserved structure of *AOX* genes consisting in 4 exons) converting an ACT to a premature stop codon (TGA) in the 85 amino acid of peptide. The predominant consequence of nonsense mutations is not the synthesis of truncated proteins, but the recognition of nonsense transcripts and their efficient degradation by a phenomenon called nonsense-mediated RNA decay (NMD) (Conti and Izaurralde 2005). This mechanism seems to guarantee that only full-length proteins are produced (Byers 2002). Frischmeyer and Dietz (1999) considered NMD as an extremely heterogenous process that might be transcript-, cell type- or genotype specific. The nonsense decay pathway participates in the control of gene expression by regulating the stability of physiological transcripts (Lew et al. 1998, Culbertson 1999). Nonsense mutations are related to a decrease of correspondent gene transcript accumulation (Nawarath et al. 2002, Aung et al. 2006). Horri and Watanabe (2007) reported that transcripts with stop codons located distant from the mRNA 3'termini or >50 nts upstream of the 3'-most exon-exon juntion are recognized as substrates for NMD. In both cultivars of olive the premature stop codon is located at 160 nts upstream the 3'-most exon-exon juntion. Nonsense mutations were related with one-third of inherited human genetic disorders

and many forms of cancer (Frischmeyer and Dietz 1999). Several authors described that the phenotypic severity of selected diseases caused by nonsense mutations can be predicted by the extent of reduction in the level of mRNA from the mutant allele (Dietz et al. 1993, Hall and Thein 1994). In plants there are several examples showing that the nonsense mutation in specific genes are related to phenotype variations (Olsson et al. 2004, Aung et al. 2006, Sattler et al. 2009).

Introns are known to participate in NMD (Frischmeyer and Dietz 1999) as a mechanism of gene expression control. This knowledge confirmed the important role of plant introns in the control of gene expression (Rose 2002, Gianì et al. 2003, Fiume et al. 2004) and contributed that recently introns are gaining new credit in the scientific community (Rodriguez-Trelles et al. 2006, Roy and Gilbert 2006).

Pre-mRNA can influence splicing decisions and induce either exon skipping or intron retention (Aoufouchi et al. 1996, Valentine 1998). There are a number of *cis* elements located in exons and introns known as exonic or intronic splicing elements or silencers, due to their stimulating or repressing effects, respectively (Liu et al. 1998, Ladd and Cooper 2002). Single base changes that affect splicing can have dramatic effects on gene function and consequently in the phenotype, usually because the splice mutation results in a shift in the amino acid reading frame. SNPs located at introns and exons were related with alternative splicing (Kawase et al. 2007, Seli et al. 2008) with a strongest correlation were those closest to the intron-exon boundaries of the splicing events (Hull et al. 2007). Thus the two SNPs leading to non-synonymous translation at positions nearby the exon-intron (SNP at position 399) and intron-exon boundaries (SNP at position 429) that were only identified in 'Galega vulgar' can be of interest for future studies. The effects of polymorphisms on splicing may represent an important mechanism by which SNPs influence differential gene function.

Defective splicing can be also related with nonsense mutations (Aoufouchi et al. 1996), which can be caused by SNPs (Isshiki et al. 2001, Nawrath et al. 2002, Aung et al. 2006, Sattler et al. 2009) or InDel events (Olsson et al. 2004).

The capacity of introns to regulate gene expression is related to intronic regulatory elements, such as miRNAs which inhibit translation of target genes by binding to their mRNAs. Recently discovered, the miRNAs have emerged as important

players in plant stress responses, playing vital roles in plant resistance to abiotic as well as biotic stresses (Chiou et al. 2006). The control of plant developmental processes has been related with miRNA, including regulation of root growth (Wang et al. 2004), leaf development (Mallory et al. 2004), flower development and fertility (Achard et al. 2004). In two sequences of 'Cobrançosa' a putative miRNA precursor sequence (pre-miRNA) was located in a region characterized by a SNP at position 703 (G/C) combined with a SNP at the position 754 (T/G). This is an example how a polymorphism can influence prediction of regulatory sequences. A similar example, where predictability of a pre-miRNA site was also influenced by DNA polymorphism, was reported for intron 3 of the carrot *AOX2a* gene (Cardoso et al. 2009) and intron 1 of St John's Wort *AOX1b* (Ferreira et al. 2009). In both cases the existence of an InDel event was related with the predictability of the pre-miRNA site. The results need to be validated in future experiments.

Variation in the 3'-UTR region is not restricted to nucleotide polymorphisms but also encompasses length polymorphisms. Examining the 3'-UTR structure in 'Galega vulgar' micro shoots a heterogeneity in 3'-UTR size was revealed due to both local microheterogeneity and alternative polyadenylation. Microheterogeneity, probably caused by polymerase slippage, could be considered in the case of the length variation from 297 to 301 nucleotides in the 3'-UTRs of 5 clones in sequences Gal_2, Gal_3 and Gal_4 (Table 4). Alternative polyadenylation could be considered the variance in 3'-UTR size among the group of these three and the rest of the other sequences. All required polyadenylation signals could be identified (Fig. 9). In the shortest 3'-UTR sequence (Gal_1) a typical cleavage site is missing that was found in all other 3'-UTRs. The presence of 5 classes of alternative polyadenylation in *OeAOX2* in a single cultivar raises the possibility for differential regulation of this gene in any given tissue or organ (Polidoros et al. 2009). 3'-UTRs play an important role in post-transcriptional regulation known to be mediated by miRNAs in animals (Stark et al. 2005). In plants miRNA sites exist anywhere along the target mRNA (Zhang et al. 2006). However, several examples exist also in plants where the miRNA target is located in the 3'-UTR (Rhoades et al. 2002). In maize *AOX1a* 3'-UTR a putative miR163 target motif was identified (Polidoros et al 2009). The maize *AOX1a* is transcribed with different 3'-UTR

length and two major classes, a shorter and a longer (Polidoros et al. 2005). The miR163 target motif is present only in the longer class. Although the functional significance of this motif in maize *AOX1a* is obscure, its differential presence in the maize *AOX* 3'-UTR can be suggested how modulation of the 3'-UTR length can have significant effects of the regulation of *AOX* genes. A search for miRNA sites in olive 3'-UTR revealed 5 putative miRNA targets that had overall pairing energy ΔG<-20 Kcal/mol. Three of these targets were present in all seven variants but the other two were absent in the shorter three variants. The functional significance of these sites remains to be examined. However, discovery of *AOX* 3'-UTR microheterogeneity in two phylogenetically distinct plant species strengthens the possibility that this phenomenon is widespread in plant *AOX* genes. There are several methodologies for biological validation of predicted miRNA targets which can be applied in the future, which include reported-gene constructs, mutation studies, gene-silencing techniques, rescue assays and classic genetic studies.

Polyadenylation requires two major components: the cis-elements or polyadenylation signals of the pre-mRNA, and the trans-acting factors that carry out the cleavage and addition of the poly(A) tail at the 3'-end (Loke et al. 2005). Analyses of *OeAOX2* transcripts with different 3'-UTR length has been restricted to a single olive cultivar and the observed variation could be correlated with the presence of the respective polyadenylation signals in the correct position (Fig. 9). It is currently not known if the structure of the *OeAOX2* 3'-UTR is conserved in other cultivars but it is conceivable that sequence polymorphisms that affect the polyadenylation signals could result in differences of transcript 3'-UTR lengths among different cultivars. This may provide an additional source of genetic variation that can be exploited in the development of a marker-assisted strategy for breeding purposes.

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Chapter 4 Adventitious root formation in olive (*Olea europaea* L.) microshoots: anatomical studies

This chapter refers to manuscript and was extracted only the important content to this thesis

Macedo ES, Vieira CM, Carrizo D, Porfírio S, Hegewald H, Arnholdt-Schmitt B, Peixe A (2013) Adventitious root formation in olive (*Olea europaea* L.) microshoots: anatomical evaluation and associated biochemical changes in peroxidase and polyphenol oxidase activities. Journal of Horticultural Science & Biotechnology 88 (1) 53–59).

4.1. Summary

Trials were performed using *in vitro*-cultured microshoots of the olive (*Olea europaea* L.) cultivar 'Galega vulgar' as initial explants, to identify histological events. Explant bases were submitted to a 10 s quick-dip treatment to promote rooting, using a sterile solution of 14.7 mM indole-3-butyric acid (IBA). Samples for histology were collected on pre-established periods from 0 to 30 days. The first signs of modifications in stem cell morphology were observed 4 days after explant inoculation on olive culture medium (OM), with some cortex cells presenting a dense cytoplasm and a large central nucleus with visible nucleoli. The first mitotic events were observed after 6 days and evolved via two different pathways; non-specific cell division, leading to callus formation, and organised cell division, leading to the formation of root meristemoids. After 19 days, the first organised root primordia became visible. No root formation was achieved without the earlier calli development and 89% of root primordia originated from tissues other than cambial/phloem.

4.2. Introduction

Considerable progress has been made in the last 20/30 years towards understanding rooting by characterising it as an evolutionary process, consisting of a successive series of interdependent phases (i.e., induction, initiation, and expression), each having specific physiological and environmental requirements (Moncousin et al. 1988, Gaspar et al. 1992, Rout et al. 2000).

Adventitious roots originate from the redifferentiation of several cell types such as those from sub-epidermal tissues, cortex, cambium, secondary phloem, pericycle, or vascular bundles. In olive, the capacity for the development of adventitious roots has proved to be extremely variable among cultivars (Salama et al. 1987, El-Said et al. 1990, Fouad et al. 1990). Differences in the anatomical structure of cuttings were proposed to explain this dependence on genotype, with several authors stating that the presence of a continuous ring of sclerenchyma, between the phloem and the cortex, may act as a mechanical barrier for root emergence (Salama et al. 1987, Qrunfleh et al. 1994). Nevertheless, other reports provided evidence that the difficulty in rooting olive cuttings could not be correlated with the anatomical structure of the cutting, and that genetic, biochemical, or physiological causes, rather than anatomical ones, could be related to the incapacity of several olive cultivars to form adventitious roots (Bakr et al. 1977, Fabbri 1980).

Despite all the research over the past 20/30 years aimed at a better understanding of adventitious rooting, the process is far from being resolved, especially in recalcitrant genotypes. The present study aimed to provide more updated information on the anatomical events during *in vitro* adventitious root formation on explants of the difficult-to-root olive (*Olea europaea* L.) cultivar 'Galega vulgar'.

4.3. Materials and Methods

4.3.1. Plant material, rooting procedure and culture conditions

Microshoots of a single clone (1053) of the olive (*Olea europaea* L.) cultivar 'Galega vulgar' already established *in vitro* according to the protocol of Peixe et al. (2007) were used in all these experiments.

Explants with four-to-five nodes were prepared from *in vitro*-cultured microshoots, and all leaves, except for the upper four, were removed. To induce rooting, explants bases (approx. 1.0 cm) were submitted to a 10 s quick-dip treatment in a sterile solution of 14.7 mM IBA. The explants were then inoculated, *in vitro*, in 500 ml glass flasks containing 75 ml semi-solid olive culture medium (OM) (Rugini 1984), devoid of plant growth regulators and supplemented with 7 g l^{-1} commercial agar-agar, 30 g l^{-1}

D-mannitol and 2 g l^{-1} activated charcoal, all supplied by Merck-Portugal, Lisboa, Portugal. The pH of the medium was adjusted to 5.8 prior to sterilisation in an autoclave (20 min at 121ºC). All cultures were kept in a growth chamber at 24ºC / 21ºC (± 1ºC) day/night temperatures and with a 15 h photoperiod, under cool-white fluorescent lights at a Photosynthetically Active Radiation (PAR) of 36 µmol m^{-2} s^{-1}.

4.3.2. Histology

During rooting, ten samples from the basal portion (approx. 1 cm from explant base) of *in vitro*-cultured explants were collected at 0h, 4h, 8h, 24h, 2days, 4days, 6days, 8days, 10days, 14days, 18days, 22days, 26days and 30days after auxin treatment. Samples were fixed in 1: 1: 8 (v/v/v) formaldehyde: acetic acid: 70% (v/v): ethanol (FAA). Each sample was placed individually in a small plastic tube (10 ml) and kept uncovered in a vacuum chamber for 1 h. The tubes were then closed and the samples were left in the fixative for 2 days at 4^{o}C. After fixation, samples were washed twice in 70% (v/v) ethanol, dehydrated through a graded ethanol series and increasing butanol solutions (Table 1), cleared in xylene and embedded in paraffin according to the procedure of Johansen (1940).

Low melting-point (56ºC) paraffin (Jung-Histowax, Cambridge Instruments, Nussloch, Germany) was used, and paraffin blocks were prepared using Leuckart's bars.

TABLE I

Dehydrating solutions (values to prepare 100 ml of each solution)

Solution	H_2O (ml)	Ethanol (ml)	Butanol (ml)	Eosin (mg)	Time (h)
I	50	40	10		4
II	30	50	20		12
III	15	50	35		2
IV		45	55	25	2
V		25	75	25	2
VI		5	95		12

Thick (10-15 µm) serial transverse sections were cut on MicroTec-Cut 4055 rotary microtome (microTec Laborgeräte GmbH, Walldorf, Germany), fixed on microscope slides covered with a thin film of Haupt's adhesive and air dried overnight at room temperature. Sections were stained with 0,6% Safranin O + 2% Orange G and observed under an Olympus CK-40 inverted optical microscope (Olympus-Portugal, Lisboa, Portugal) equipped with a 50 watt mercury arc lamp fluorescent unit, with a green light filter cube U-MWG (510-550 nm excitation filter, 590 nm emission filter and 570 nm dichromatic mirror). With this filter combination lignin and Safranin O stained cells organelles, should present light red fluorescence.

4.4. Results

4.4.1. Anatomical observations

The sequence of events leading to the formation of adventitious roots in *in vitro*-cultured explants of the olive cultivar 'Galega vulgar' is described. The moment in time presented for each histological event corresponds to its first identification on the stem samples under observation, because these events were not synchronous in all examined samples.

A transverse section of the stem-base, before being submitted to IBA treatment, is presented in Figure 1, Panel A. A collateral vascular bundle forming a ring around the pith, which is a typical feature for dicotyledonous species, can be observed. The cambial zone is represented by a few layers of flat cells between the xylem and the phloem. The epidermis is formed by one or two cell layers, whereas the cortex consists of several layers of large parenchymatous cells. The evolution of the stem-base tissues 4 days after IBA treatment can be observed in Figure 1, Panel B. Cells randomly distributed both in the cortex and sub-epidermal tissues reacquired characteristics of meristematic cells, presenting dense cytoplasm, large centrally positioned nuclei, and prominent nucleoli.

The first cell divisions were identified at 6 days after root inducing treatment and two developmental pathways have been observed. The first one, following an unorganized pattern of cell divisions, led to the formation of the cicatricial calli (Fig. 1 – Panels C and C*), while the second one, involved organised divisions of isodiametric cells, leading to the development of meristemoid regions.

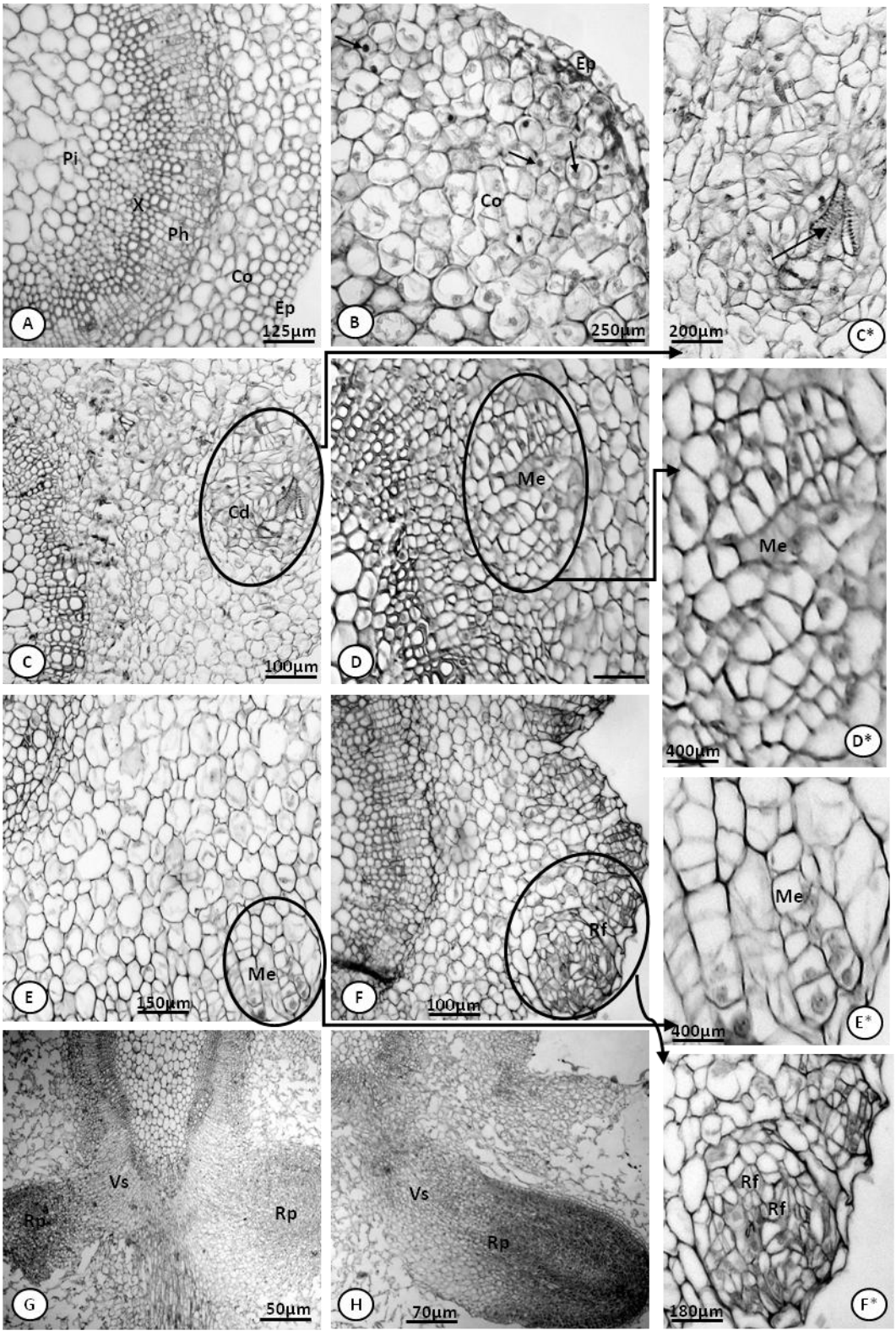

Fig. 1. Sections of the stem basal region, at root origin, 0-30days after IBA root inductive treatment. Panel A, anatomical structure of the stem-base before root-induction treatment, showing a vascular bundle; Pi, pith; Co, cortex; Ep, epidermis; Ph, phloem; X, xylem. Panel B, transverse section near the stem-base at 4 days. Cells in the cortex reacquire meristematic

characteristics, presenting a dense cytoplasm with large nuclei and visible nucleoli (arrows); Ep, Epidermis; Co, Cortex. Panel C, first cell divisions (Cd) at 6 days, leading to calli formation. Magnification of the circular region is presented in Panel C*. Xylem tracheids (arrow) are also visible. Panels D and E, stem sections after 10 days on rooting medium showing two meristemoid structures (Me) in the upper phloem/cortex in Panel D and in the sub-epidermal region in Panel E. Magnifications of the circular regions are presented in Panels D* and E* respectively. Panel F, morphogenic root fields (Rf) developing from sub-epidermal cells 14 days after root induction treatment. Magnification of the circular region is presented in Panel F*. Panels G and H, root primordia (Rp) at different developmental stages, 22 days after root induction treatment. The root caps (Rc) and differentiated the vascular system (Vs) can be observed.

These meristemoids, developing from the upper phloem (Fig. 1 - Panels D and D*) and from the cortex/sub-epidermal region (Fig. 1 – Panels E and E*), were first observed 10 days after the IBA root induction treatment. The most responsive region was the cortex/sub-epidermal one, where 89% of the total root meristemoids were formed. The first morphogenetic root fields resulting from synchronised divisions of meristemoid cells were observed at 14 days (Fig. 1- Panels F and F*) and, root primordia, exhibiting an evident polarisation due to the presence of root meristem and a differentiated vascular system connected to that of the stem, were visible 22 days after the root induction treatment was applied (Fig. 1- Panels G and H).

Using the fluorescence filters combination previously presented in Materials and Methods, it was possible to easily identify stem regions where mitotic activity and lignin deposition is occurring. Figure 2, Panel A, shows a stem section sampled before root inductive treatment. Fluorescent cell walls were only observed in xylem and in some suberised epidermal regions, with all the other stem tissues displaying no fluorescent signal, indicating the absence of mitotic activity or lignin deposition. A stem section 10 days after explants inoculation in rooting medium is presented in Figure 2, Panel B. Most cell walls display red fluorescence due to lignin deposition while light red nucleoli can be observed in regions where cell mitosis is taking place. The image corresponds to the one previously presented in Figure 1, Panel D clearly

proving the efficiency of the observation under florescent light to identify the mitotic events during adventitious root formation.

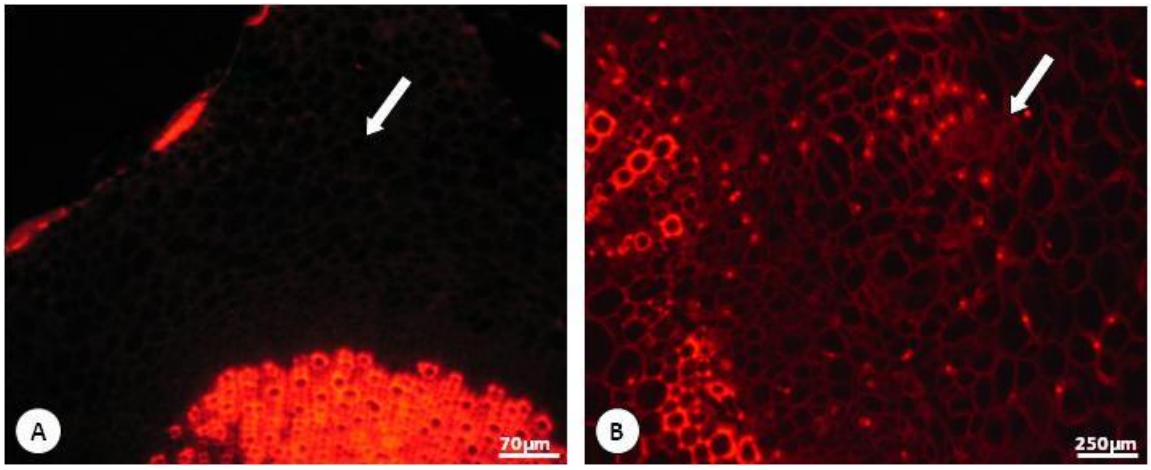

Fig. 2. Sections of the stem-base region (at different stages of adventitious root formation) observed under fluorescent light. Panel A, transverse section of the stem-base before root induction treatment. Arrow indicates the cortex region where no fluorescence signal can be observed due to absence of mitotic activity or lignin deposition. Panel B, transverse section of a stem-base 10 days after root induction treatment was applied. High rate of mitotic events can be observed in the upper-phloem and cortex regions, where cell nuclei and nucleoli, as well as lignin in cell walls, exhibit intense fluorescence (arrow). The image presented in Panel B corresponds to the one presented in Figure 1, Panel D, where it was observed without fluorescent lighting.

4.5. Discussion

The present work allowed to conclude that before root induction, the stem structure observed in microshoots cultured *in vitro* was basically the same as that described for semi-hardwood olive cuttings (Troncoso et al. 1975, El-Nabawy et al. 1983, Ayoub and Qrunfleh 2006). However, we did not observe the sclerenchyma ring reported by the aforementioned authors in *in vitro*-cultured microshoots, probably due to the softness of the stem tissues used.

The first mitotic events observed in the cultured explants led to the formation of the cicatricial calli. According to Hartmann et al. (1997), callus formation prior to rooting normally occurs during indirect root formation and represents a common feature in difficult-to-root explants. In our trials with the olive cv. Galega vulgar, calli

arose from the cortical cells, which is in accordance with observations made by Ayoub and Qrunfleh (2006) working with semi-hardwood cuttings of the olive cultivars 'Nabali' and 'Raseei'.

Despite some differences in timing, all other stages of adventitious root formation (e.g., the development of root meristemoids, evolution into morphogenic roots and the emergence of root primordia) also agreed with the results of similar *in vitro* studies in other temperate fruit species (e.g. *Malus pumila* 'KSC-3' (Hicks 1987), *Prunus avium* L. × *Prunus pseudocerasus lind* (Ranjit et al. 1988) and *Castanea sativa* L. (Gonçalves et al. 1998).

The major difference between our results and those reported by other authors concern the stem tissues involved in the formation of a new adventitious root system. For olive cuttings, independently of the rooting ability of the cultivar, most authors observed the adventitious roots arising from the cambial region of the stem; Bakr et al. (1977) on cultivar 'Wetaken', Salama et al. (1987) on 'Manzanillo', 'Mission', 'Calamata' and 'Hamed' and Ayoub and Qrunfleh (2006) on Nabali' and 'Raseei'. However in this study, we did not observe adventitious roots arising from the cambial region in the olive cv. Galega vulgar. Nevertheless, as stated by Naija et al. (2008), the region in which cells become activated seems to depend, in part, on physiological gradients of substances entering the shoot from the medium, and on the presence of competent cells to respond to the inductive stimulus.

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Chapter 5 Involvement of alternative oxidase (AOX) in adventitious rooting of *Olea europaea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism

This chapter was extracted from the following manuscript but contains additional experiments (*):

Macedo ES, Sircar D, Cardoso HG, Peixe A, Arnholdt-Schmitt B (2012) Involvement of alternative oxidase (AOX) in adventitious rooting of *Olea europaea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism. Plant Cell Reports 31(9): 1581-90.

5.1. Summary

Alternative oxidase (AOX) has been proposed as a functional marker candidate in a number of events involving cell differentiation, including rooting efficiency in semi-hardwood shoot cuttings of *Olea europaea* L.. To ascertain the general importance of AOX in olive rooting, the auxin-induced rooting process was studied in an *in vitro* system for microshoot propagation. Inhibition of AOX by salicylhydroxamic acid (SHAM) significantly reduced rooting efficiency, which was confirmed by a histological analysis showing the inhibition of morphogenic fields development. However, the inhibitor failed to exhibit any effect on the preceding calli stage. This makes the system appropriate for distinguishing dedifferentiation and *de novo* differentiation during root induction. Metabolite analyses of microshoots showed that total phenolics, total flavonoids and lignin contents were significantly reduced upon SHAM treatment. It was concluded that the influence of alternative respiration on root formation was associated to adaptive phenylpropanoid and lignin metabolism. Transcript profiles of two olive *AOX* genes (*OeAOX1a* and *OeAOX2*) were examined during the process of auxin-induced root induction. Both genes displayed stable transcript accumulation in semi-quantitative RT-PCR analysis during all experimental stages. In contrary, when the reverse primer for *OeAOX2* was designed from the 3'-UTR instead of the ORF, differential transcript accumulation was observed suggesting posttranscriptional regulation of *OeAOX2* during metabolic acclimation. This result confirms former observations in olive semi-hardwood shoot cuttings on differential *OeAOX2* expression

during root induction. It further points to the importance of future studies on the functional role of sequence and length polymorphisms in the 3'-UTR of this gene.

5.2. Introduction

Olive (*Olea europaea* L.) is one of the oldest agricultural tree crops in the world and the source of olive oil which possesses health promoting properties (Bracci et al. 2011). Olive trees show high variation in rooting efficiencies and some valuable cultivars suffer from rooting rates below 20%. Genotypic differences can be explored for the identification of functional marker candidate genes that can assist tree breeding for higher rooting rates. How genetic variation affects capabilities for rooting is unknown. Olive rooting can be seen as stress-induced reprogramming of shoot cells (Macedo et al. 2009).

The plant mitochondrial electron transport chain is characterized by presence of alternative oxidase (AOX) that competes for electrons with the standard cytochrome (Cyt) pathway (Fiorani et al. 2005). AOX branches from the main respiratory chain at the level of the ubiquinone pool and catalyzes the four-electron reduction of oxygen to water without any ATP production. Thus AOX can use excess reductant from the Cyt pathway and thereby plays a central role in determining reactive oxygen species (ROS) equilibrium in plants (Amirsadeghi et al. 2006). *AOX* is known to be induced in response to diverse abiotic and biotic plant stress conditions or respiratory chain inhibition (Vanlerberghe and McIntosh 1996). Growing evidences suggest that under stress conditions plant mitochondria generate ROS which convey to nucleus as a signal to induce the transcription of genes such as *AOX*, whose products are needed to cope with altered metabolic conditions (Maxwell et al. 1999) leading to enhanced stress tolerance capacity (Dutilleul et al. 2003). It is likely that AOX may be to modulate the strength of a stress-signaling pathway from the mitochondrion that controls cellular responses to stress and thus maintain signaling homeostasis from the mitochondrion (Vanlerberghe et al. 2009). Based on recent development on AOX research it is presumed that AOX plays a key role in regulating the process of cell-reprogramming by ameliorating metabolic transitions related with the cellular redox state and the flexible

carbon balance (Arnholdt-Schmitt et al. 2006a, Rasmusson et al. 2009), however the physiological and molecular basis of such regulation remain largely unknown. Although in most plant species *AOX* is expressed during normal growth and development (Clifton et al. 2007), it is often dramatically induced during various stress conditions (Giraud et al. 2008, Li et al. 2012, Liao et al. 2012,) and during the process of cell-reprogramming (Campos et al. 2009, Frederico et al. 2009). Supplementing salicylhydroxamic acid (SHAM), a known inhibitor of AOX, can suppress various cell-reprogramming processes such as realization of somatic embryogenesis in *Daucus carota* (Frederico et al. 2009), rhizogenesis in *Olea europaea* L. (Santos Macedo et al. 2009), and in *Helianthus tuberosus* (Hase 1987). Interestingly, the influence of AOX in cell-reprogramming can be observed not only in plants but also in fungi as demonstrated during mycelial to yeast differentiation in *Paracoccidioides brasiliensis* (Martins et al. 2011). Despite the amount of data concerning transcript accumulation of *AOX* genes during various cell-reprogramming processes, little is known about the role of AOX in metabolic reprogramming *in planta* (Moore et al. 2002).

Phenylpropanoid derivatives, especially phenolic acids and lignin are known to be crucially involved in regulating cell-division and differentiation (Tamagnone et al. 1998). It has been observed that reduced phenolic acid and lignin biosynthesis inhibits cell division and differentiation process (Cvikrová et al. 2003, Palama et al. 2010). Enhanced accumulation of phenolic acids and flavonoids was found to be highly stimulating for *in vitro* rooting (Fu et al. 2011). Phenolic acids, flavonoids and lignin are derived from the shikimate pathway through the common phenylpropanoid pathway (Boudet et al. 1995). Substantial allocation of carbon-energy and reducing power is required for its formation (Booker and Miller 1998). The ability of AOX to affect redox and energy metabolism may serve to maintain energy charge and constant growth under a variety of changing circumstances (Moore et al. 2002).

AOX was proposed as functional marker candidate for efficient adventitious rooting of olive (*Olea europaea* L.) (Arnholdt-Schmitt et al. 2006b, c). Recently, Santos Macedo et al. (2009) showed that SHAM treatment significantly reduced the number of rooted semi-hardwood shoot cuttings in the easy-to-root cv. Cobrançosa and differential expression of *OeAOX2* gene was observed during auxin-induced root

formation. To study the general role of AOX in olive rooting, analyses were performed in an *in vitro* system developed for easy propagation of the Portuguese olive cv. Galega vulgar for commercial purposes (Peixe et al. 2007). Cv. Galega vulgar is an important cultivar in Portugal that demonstrates between 5-20% rooting in semi-hardwood shoot cuttings. However, under optimized *in vitro* conditions microshoots can achieve 60-90% rooting (Peixe et al. 2010). Our results on microshoots and histological analysis confirm the general effect of SHAM to suppress adventitious root formation in olive. We report the inhibition of phenylpropanoids and lignin metabolism after SHAM treatment during the process of auxin-induced root formation in cv. Galega vulgar under *in vitro* conditions. In addition, studies on transcript accumulation of two olive *AOX* genes (*OeAOX1a* and *OeAOX2*) during the process of root induction will be presented.

5.3. Materials and Methods

5.3.1. Plant material

Olea europaea L. microshoots of cv. Galega vulgar (clone 1441) provided by the Superior Agricultural School of Santarem (Portugal) were used to establish *in vitro* cultures by micropropagation according to Peixe et al. (2007). The derived *in vitro* grown plantlets were used to establish the following assays.

5.3.2. Analysis of SHAM effects on adventitious rooting

Indole-3-butyric acid (IBA; Sigma-Aldrich, St. Louis, MO, USA) was used as root promoting auxin. The basal part of microshoots (±1 cm) with 3 to 5 nodes containing a maximum of 4 upper leaves were dipped for 10 s into IBA solution (14.7 mM) and then quickly transferred into rooting medium (Peixe et al. 2007). To study the involvement of AOX in adventitious rooting, SHAM (Sigma-Aldrich, St Louis, MI) was used as AOX inhibitor. Different SHAM concentrations (1, 10 and 100 mM) were applied for 10 s either with or without previous treatment of IBA. SHAM stock solution was prepared in dimethyl sulfoxide (DMSO, Fluka, France) and used freshly. The maximum DMSO concentration used to dissolve highest SHAM concentration was 26% (v/v). Inhibitory

effect of DMSO on rooting was previously tested using IBA-treated microshoots dipped into various concentrations of aqueous DMSO solution (0.8% to 26% DMSO; v/v) and then transferred to rooting medium. The cultures were kept in a growth chamber at 24±1 ^{o}C day and 21±1 ^{o}C night temperature, and 15 h photoperiod under cool white fluorescent light (36 µmol m^{-2} s^{-1}).

In each treatment, 10 microshoots were taken and at least 4 repetitions were performed. In all experiments visible calli and root formation was recorded 56 days after transferring into rooting medium. All data in percentage was transformed in *(arc sen* $\frac{\sqrt{x}}{100}$*)* prior to ANOVA analyses and post-hoc tests.

5.3.3. Histological analysis of SHAM effects on adventitious rooting (*)

In order to investigate the affect of AOX in the development of morphonenic fields and adventitious rooting development an AOX inhibition study was carried out using following treatment: (i) IBA (14.7 mM) (positive control); (ii) IBA (14.7 mM) and SHAM (200 mM). For the experiment establishment *in vitro* cultured 'Galega vulgar' microshoots with 4-5 nodes and 2-4 apical leaves were used. The basal part of microshoot (± 0.5 cm) was dipped for 10 s in each sterile solution and immediately inoculated into rooting medium (Peixe et al. 2007). Cultures were kept at ± 25^{o}C and 16h photoperiod. Three samples from the basal part of the *in vitro* cultured microshoots were collected at 14 and 18 days after inoculation and fixed in FAA (1 formaldehyde : 1 acetic acid: 8 ethanol 70%). For each time point were collect samples from three explants. For samples dehydration and paraffin embedding was followed the protocol previously described in Chapter. 2. Transverse sections (10-15 µm thick) obtained using a rotary microtome (Micro Cut Tec 4055) were fixed on microscope slides, covered with a thin film of Haupt's adhesive and incubated overnight at room temperature. After dry, the sections were stained after wax removal with Safranin O + Orange G. Sections analysis was performed using an Olympus CK-40 optical microscope equipped with a fluorescent light unit using the exciter filter BP 460-490 nm and the barrier filter OG 515IF. As described in Chapter. 4 the use of this filter facilitate the

visualization of lignin deposition since lignin presents autofluorescence under this specific wave lengths.

5.3.4. Analyses of phenolics, flavonoids and lignin content

In order to investigate the affect of AOX in phenylpropanoid metabolism, AOX inhibition studies were carried out using following treatments: (i) DMSO in the same concentration that had been used to dissolve IBA and SHAM (negative control), (ii) IBA (14.7 mM) (positive control); (iii) IBA (14.7 mM) and SHAM (200 mM). Induction of rooting was performed in microshoots following the procedures descrived before. To determine total phenolics, flavonoids and lignin content the basal part (± 1 cm) of 40 microshoots (0.5 g) was collected as bulked sample at different time points: 0, 12 and 25 d after treatment. The assay was repeated three times, thus, inoculating in total 120 microshoots per treatment.

To quantify the total amount of phenolics the plant material was crushed in liquid nitrogen and then extracted with 3 mL of 50% (v/v) methanol at room temperature. The suspension was homogenized for 5 min and then centrifuged at 5000 rpm for 15 min. The supernatant was collected and used as extract to determine total soluble phenolics and total flavonoids. Total phenolic content of microshoots were measured using Folin and Ciocalteu method (Chakraborty et al. 2008). Briefly to 0.1 mL of extract, 0.5 mL Folin-Ciocalteau reagent (dilution 1:9) was added and incubated at room temperature for 5 min to initiate the reaction; after that 0.4 mL of 5% sodium carbonate was added, and the whole mixture was incubated at room temperature for further 20 min. The absorbance was measured at 765 nm using a NanoDrop-2000C (Thermo Scientific, Wilmington, DE, USA) spectrophotometer. Total phenolic content was expressed as milligrams of gallic acid equivalents per gram fresh weight.

The extraction and analyses of total flavonoids followed the methods reported by Wang et al. (2008). In brief, 0.5 mL ethanolic solution of 2% $AlCl_3$ was added to 0.1 mL of each sample, final volume was adjusted to 1 mL with 50% (v/v) methanol. The mixture was incubated at room temperature for 1 h and then absorbance was monitored at 420 nm. Total flavonoid content was expressed as quercetin equivalents.

Total lignin content was estimated spectrophotometrically as thioglycolic acid (TGA) derivative. Extraction and subsequent thioglycolic acid (TGA) derivatization of lignin was carried out as described by Ali et al. (2006). The amount of lignin was determined spectrophotometrically at 280 nm. The lignin content was expressed as the absorption values (A_{280}) measured at 280 nm using 1 N NaOH as the blank.

5.3.5. Transcript quantification by semi-quantitative RT-PCR

Two *OeAOX* genes (*OeAOX1a* and *OeAOX2*) were selected for transcript accumulation studies during adventitious rooting. For *OeAOX1a*, the primer pair was designed in the ORF (specific primers used in this work are listed in Table 1). For *OeAOX2* transcript analyses, two different pairs of primer sets were used. In a first set both primers were designed in the ORF and in a second set, the forward primer was designed in ORF while the reverse primer was designed in 3'-UTR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
OeAOX1a	GACATTCATTATCAGGGA	AGTATAGTTCAGACCATT
OeAOX2 (exon 3)	CCACTAGTTGTTGGTCGTGTGC	TGCTTCGTCGGCTCGGAT
OeAOX2 (3'-UTR)	CCACTAGTTGTTGGTCGTGTGC	AGTAAGTGGCATGTTTCTGTAGA
OeActin	TTGCTCTCGACTATGAACA GG	CTCTCGGCCCCAATAGTAATA

Table 1: Genes and respective primers used for SQ-RT-PCR.

Induction of rooting was performed in microshoots by using 14.7 mM IBA following the procedure previously described. *AOX* expression analyses were performed at selected time points: i) before IBA-treatment (0 h) and after IBA-treatment at 2 h, 4 h, 5 h, 12 h, 24 h, 2 days, 3 days, 6 days and 14 days for *OeAOX1a* and *OeAOX2* analysis using both primers located in the ORF; ii) 0 h, 2 h and 14 days for *OeAOX2* transcript analysis using the reverse primer located in the 3'-UTR. The basal part of four microshoots were bulked for RNA extraction and subsequent cDNA synthesis. Plant material was collected twice to perform two biological repetitions. RNeasy® plant mini kit (Qiagen, Hilden, Germany) was used for RNA extraction according to manufacturer's instruction. To eliminate contaminating genomic DNA, RNase-free DNase I (Fermentas, Ontario Canada) was additionally applied according to manufacturer's instructions. Total RNA content was measured using NanoDrop-2000C

spectrophotometer (Thermo Scientific, Wilmingtom, DE, USA) and integrity was analyzed by electrophoresis. DNase-treated RNA (2 ug) was used for cDNA synthesis with oligo d(T)18 primer using RETROscript® kit (Ambion, Austin, TX, USA) according to manufacturer's instruction. Semi-quantitative RT-PCR (SQ-RT-PCR) analysis was performed by adding 1 μL of a diluted cDNA sample (1:9) as template to the Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) as described before (Santos Macedo et al. 2009), two technical repeats were performed for each biological sample. *OeActin* transcript level was used as housekeeping. The RT-PCR products were analyzed by electrophoresis in a 1.4 % (w/v) agarose gel, stained with ethidium bromide and photographed with a Gene Flash Bio Imaging system (Syngene, Cambridge, UK). To compare the level of expression of *OeAOX* mRNAs against the *OeActin* reference gene, image analysis was employed using the image J (1.36b) [http://rsb.info.nih.gov/ij/] program. Image J program can calculate area and pixel value statistics of user-defined selections. The intensity of transcripts was evaluated numerically to compare easily.

5.4. Results

5.4.1. SHAM treatment suppresses rooting on olive microshoots

As expected, IBA treatment of olive microshoots of cv. Galega vulgar induced adventitious root formation in all assays. Root induction was always preceded by calli formation at the site of hormonal treatment (Fig. 1). A short pulse of 10 s in a solution with SHAM additionally to IBA was sufficient to reduce significantly the number of adventitious roots. In a dose-response study SHAM-mediated rooting inhibition proved to be concentration dependent with a maximum of suppressed rooting at 100 mM SHAM (Fig. 2). The possibility of toxic effects of the SHAM solvent DMSO on adventitious rooting could be excluded because microshoots simultaneously treated with IBA and DMSO didn't present a significant reduction in rooting rates, when compared with microshoots treated only with IBA (positive control), even when DMSO was applied in concentrations higher than those used to dissolve SHAM (data not presented). Also from data in figure 2 it can be seen that SHAM treatment did not

significantly inhibit calli formation, even at the highest concentrations tested. These observations argue for an involvement of AOX in root initiation, however raises at the same time the question against the involvement of AOX in the dedifferentiation process and in calli formation.

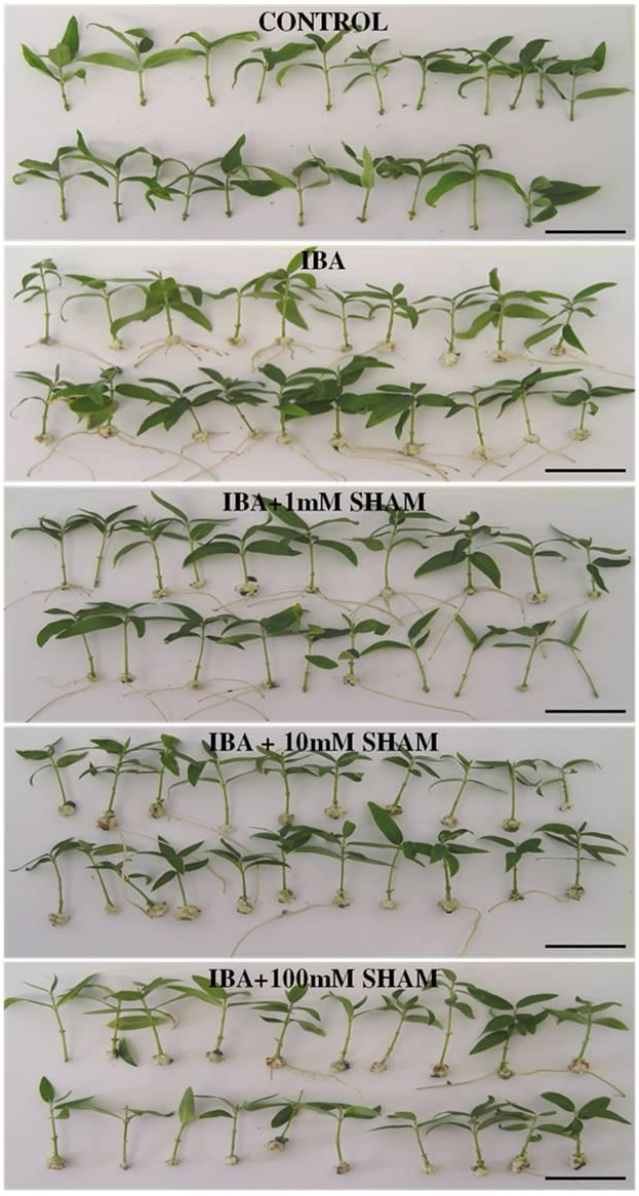

Fig. 1. Inhibitory effect of SHAM on *in vitro* rooting of *Olea europaea L.* Root inhibition rate was positively correlated to SHAM concentration. IBA was applied for 10 s in a concentration of 14.7 mM.
Bar: 4 cm

5.4.2. SHAM treatment suppresses differentiation of root morphogenic field and consequently root primordium formation on olive microshoots (*)

According with the results reported in the Chapter 4, 10 days after rooting induction organized cell divisions were observed in the cortex cells which show dense cytoplasm

and central large nuclei with visible nucleoli not visible in explants submitted to SHAM treatment (Fig. 3- C). The exposition of the same sections to a filter for lignin excitation allowed to the identification of the regions with lignin deposition, which in the case of explants submitted to SHAM treatment was mostly restricted to xylem (Fig. 3-D), and in the explants not submitted to SHAM was associated with the morphogenic fields (Fig. 3-B).

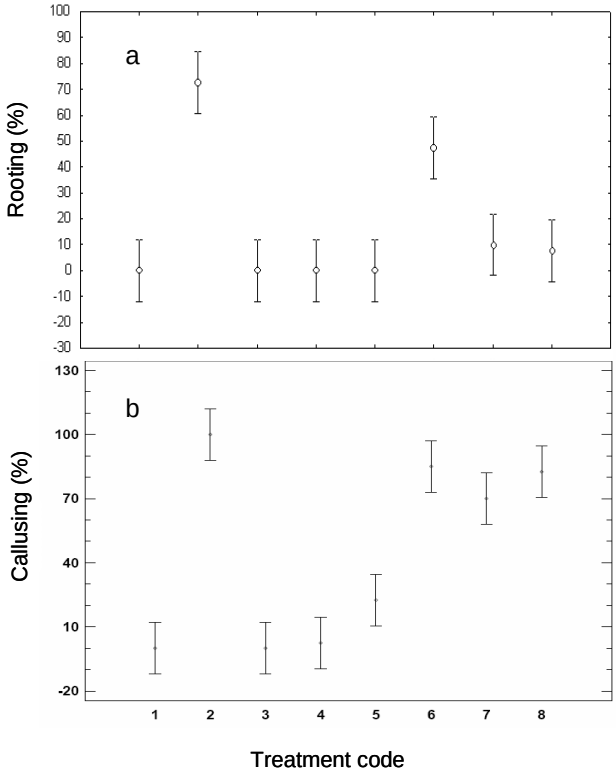

Fig 2. Rooting (a) and callusing (b) rates observed in SHAM trial (LSD 95%). Each treatment was carried out in quadruplicate sets with each set containing 10-micro-shoots.

Key to treatments identity:
1: control; **2**: IBA; **3**: 1 mM SHAM; **4**: 10 mM SHAM; **5**: 100 mM SHAM; **6**: 1 mM SHAM (+IBA); **7**: 10 mM SHAM (+IBA); **8**: 100 mM SHAM (+IBA). IBA was applied for 10 s in a concentration of 14.7 mM.

However cell activity related with non-specific cell division, leading to cicatrical calli formation was common to explants from both treatments. After eighteen days of inoculation the difference between the control (means explants not submitted to SHAM treatment) and the SHAM treatment became stronger since on the control was possible to observe the appearance of root primordial, not visible in the explants treated with SHAM (Fig. 3-E and G). At this time lignin fluorescence can be observed on the root primordium development site on explants not submitted to SHAM (Fig. 3-F). With this study was possible to clarify that the inhibition due by SHAM occurs not at

the dedifferentiation phase, since both treatments present the development of cicatricial calli, but at the morphogenic root differentiation.

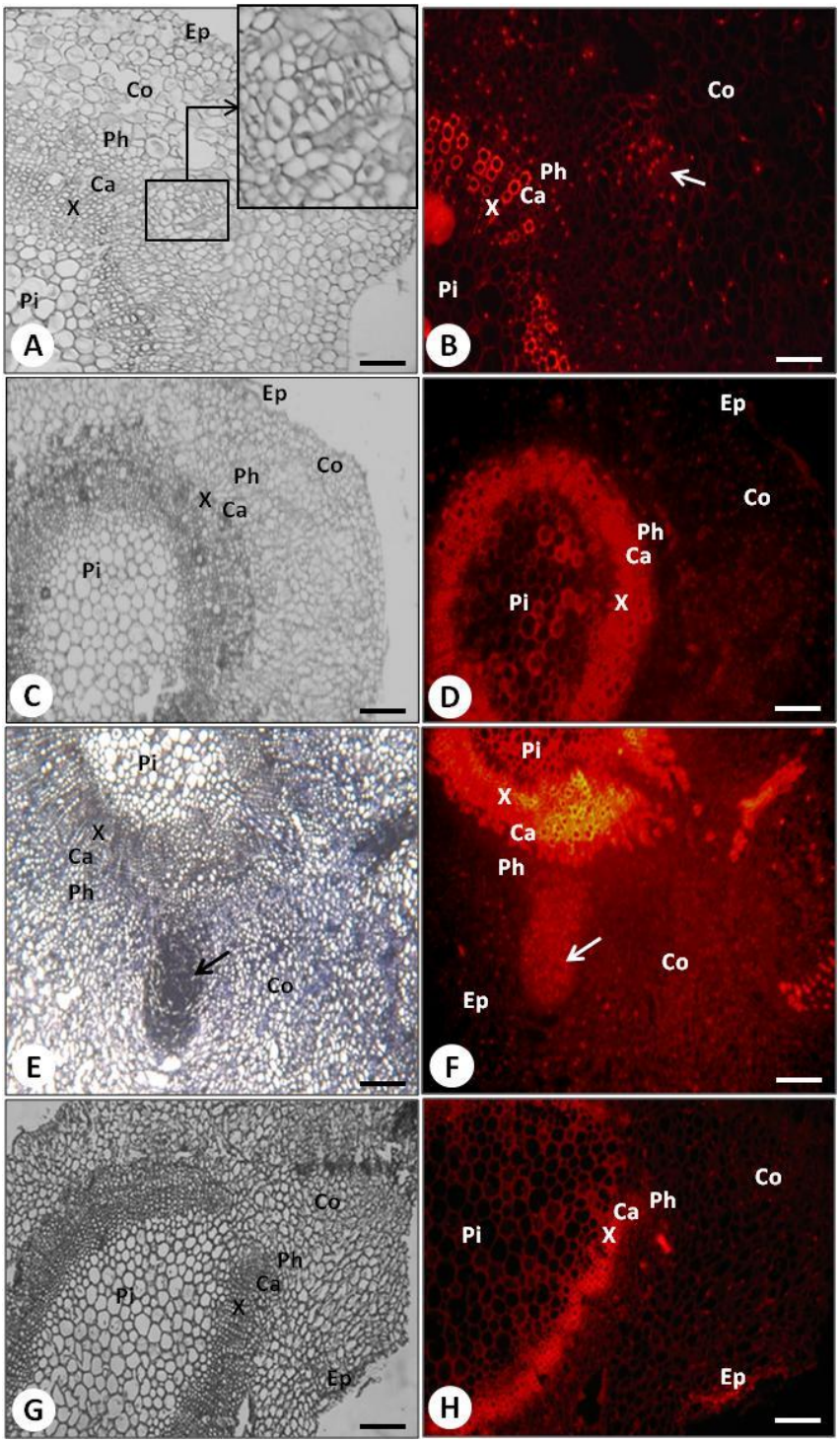

Fig. 3. Transverse sections near the stem basal region after IBA (14.7 mM) 10 s quick-deep treatment without and with SHAM treatment. 14 days after treatment: **(A)** and **(B)** in the treatment without SHAM organized cell divisions lead to the formation of morphogenic field at

the upper phloem/cortex were observed (arrow), **(C)** and **(D)** with SHAM treatment no organized cell division was observed; 18 days after treatment: **(E)** and **(F)** without SHAM was visible the appearance of root primordium (arrow) not visible with SHAM treatment **(G)** and **(H).** Pi: pith, X: xylem, Ca: cambium, Ph: phloem, Co: cortex, Ep: epiderm. *Bar:* 100 µm

5.4.3. SHAM treatment suppresses accumulation of total phenolics, total flavonoids and lignin

In order to check the influence of AOX on phenolic biosynthesis, a time-course analysis of total phenolics was carried out in IBA-treated microshoots in presence and absence of SHAM (Fig. 4). A significant increase in the accumulation of total phenolics was observed preceding root initiation in IBA-treated explants. Compared to control (0 days), 77.5% (4.5 mg/g fresh weight) and 107% (5.2 mg/g fresh weight) increase in total phenolics were observed at 12 and 25 days, respectively in IBA treated explants (Fig. 4). When SHAM was applied in conjugation with IBA, a dramatic decrease in total phenolic accumulation was observed. Compared to 0 day, only 41% (3.35 mg/g fresh mass) and 48% (3.5 mg/g fresh mass) increase in total phenolics were observed at 12 and 25 days, respectively, in explants treated with IBA and SHAM together.

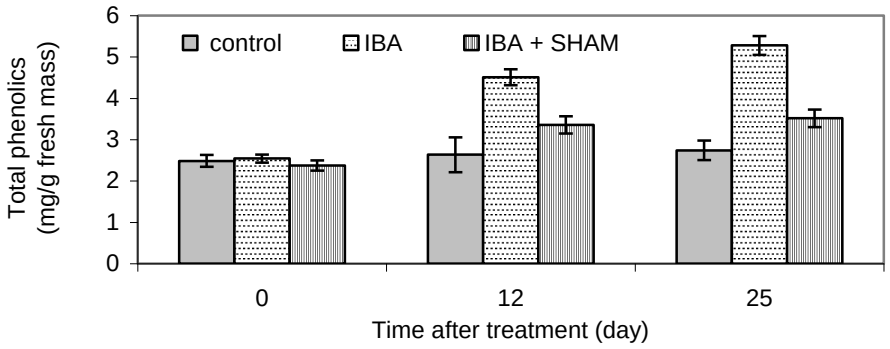

Fig. 4. Trends in total phenolic content changes in microshoots during the process of IBA-induced adventitious rooting process. Total phenolic content was expressed in milligrams of gallic acid equivalent per gram fresh mass. Values represent the average of three measurements ± SD (three biological samples each consisting of the bulked basal parts of 40 microshoots).

A parallel result was observed for total flavonoid accumulation in SHAM treated explants (Fig. 5). When IBA was applied alone, a 49% and 83% increase in total flavonoid was observed at 12 and 25 days respectively, compared to total flavonoid

content at 0 day. When SHAM was applied together with IBA, the increase in total flavonoid was only 19% (12 days) and 28% (25 days), compared to 0 days. In negative control explants no significant changes in total phenolic and total flavonoid contents were observed over the time-course studied. These results clearly indicated a positive influence of AOX on phenolics and flavonoid biosynthesis.

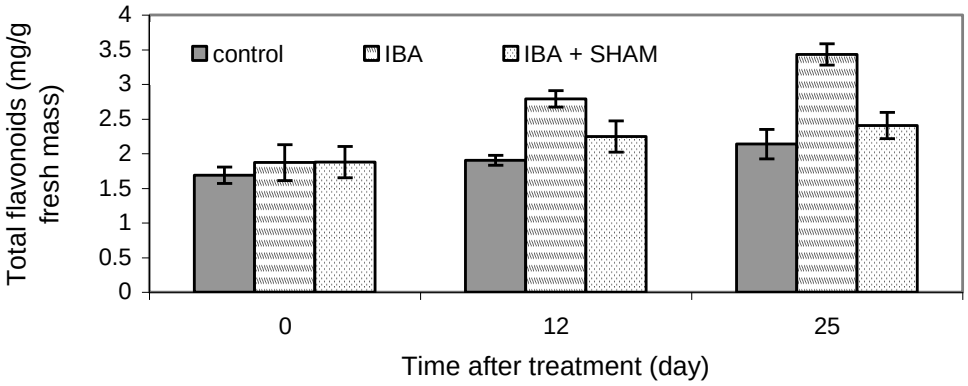

Fig. 5. Changes in total flavonoid content in microshoots during the process of IBA-induced adventitious rooting process. Total flavonoid content was expressed in milligrams of quercetin equivalent per gram fresh mass. Values represent the average of three measurements ± SD (three biological samples each consisting of the bulked basal parts of 40 microshoots).

In IBA-treated explants a signification increase in lignin content was observed preceding root induction (Fig. 6). Time-course analyses revealed that at 12 and 25 days after IBA treatment, lignin content increased to 81% and 106% respectively, compared to 0 day. SHAM treatment had an inhibitory effect on lignin accumulation. Compared to IBA treated explants, 27% and 32% decrease in lignin accumulation was observed at 12 and 25 days, respectively. Untreated explants showed no significant change in lignin content over the time course studied. This observation argues for the association of AOX activity and lignin metabolism.

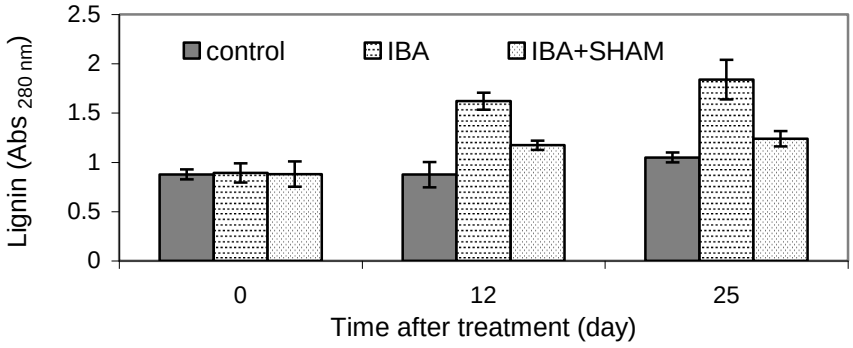

Fig. 6. Inhibitory effect of SHAM on lignin accumulation during the process of IBA-induced adventitious root formation. Values represent the average of three measurements ± SD (three biological samples each consisting of the bulked basal parts of 40 microshoots).

5.4.4. Analysis of *OeAOX1a* and *OeAOX2* transcript accumulation

Transcript accumulation of *OeAOX1a* and *OeAOX2* were studied during the process of IBA-induced adventitious rooting (Fig. 7). The amount of *OeAOX* transcripts were normalized through a ratio of integrated densities of *OeAOX* and *OeActin* amplicons. Both *OeAOX1a* and *OeAOX2* showed stable transcript accumulation during the selected time points when ORF primers were used. Transcript intensity was higher for *OeAOX1a*.

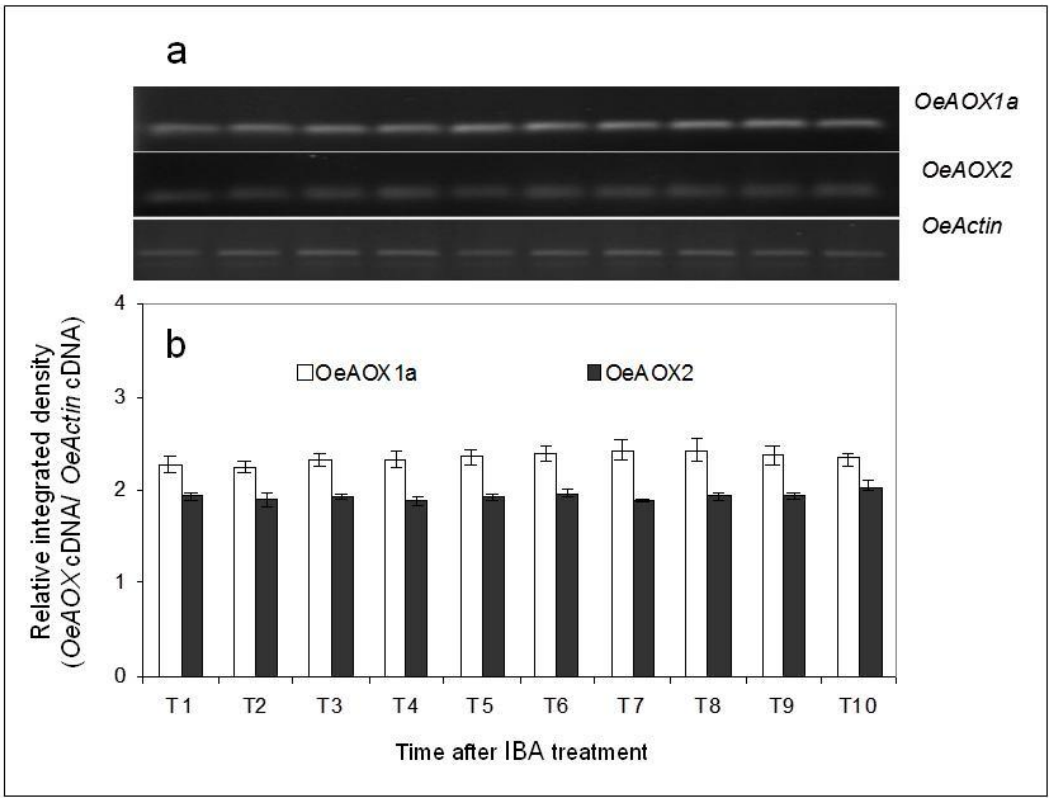

Fig. 7. Transcript levels of two *OeAOX* genes during the process of IBA-induced adventitious root formation. (a) The gel images are representatives of the experiment and show the SQ-RT-PCR of *OeAOX1a*, *OeAOX2* and actin (*OeActin*); (b) Normalization of the quantity of *AOX* transcripts through a ratio of integrated amplicon densities of the *OeAOX* and actin (*OeActin*) . Data (b) are the mean values ± SD of two biological samples (bulked basal parts of 4 microshoots) and two technical repetitions. Samples were collected before IBA-treatment (T1) and after IBA-treatment at 2 h (T2), 4 h (T3), 5 h (T4), 12 h (T5), 1 day (T6), 2 days (T7), 3 days (T8), 6 days (T9), and 14 days (T10).

When the reverse primer to amplify *OeAOX2* was located in the 3'-UTR, differential transcript accumulation was observed. The level of transcript accumulation of *OeAOX2* was dramatically increased at day 14 (Fig. 8), the time when morphogenetic changes preceding root primordia formation can be observed in microscopic analyses (data not shown).

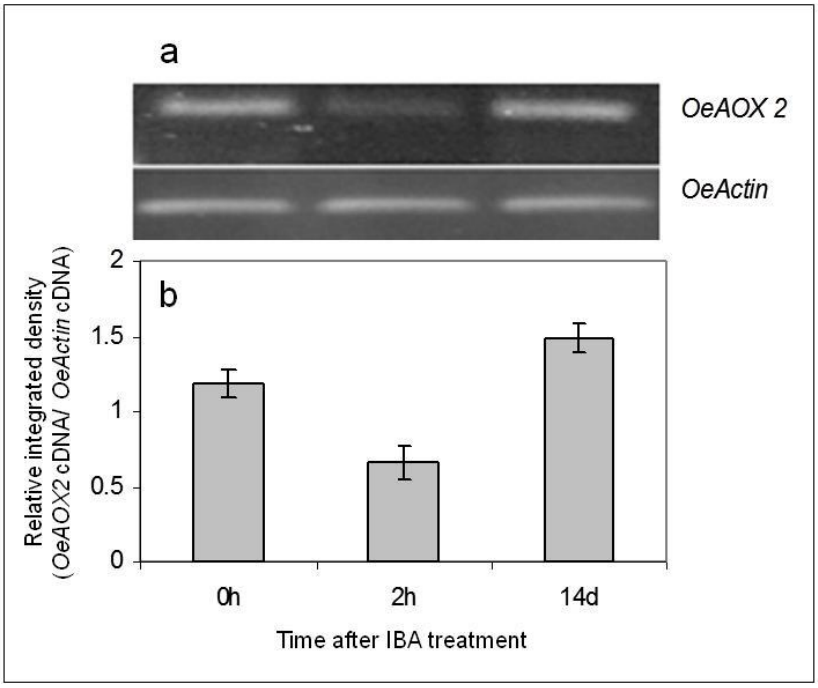

Fig. 8. Transcript accumulation pattern of *OeAOX2* gene during the process of IBA-induced adventitious root formation in *Olea europaea* L. microshoots. (a) The gel images are representatives of the experiment and show the SQ-RT-PCR of *AOX2* and *Actin* (*OeActin*); (b) Normalization of the quantity of target transcripts through a ratio of integrated densities of the *OeAOX2* and *Actin* (*OeActin*) amplicons. Data (b) are the mean values ± SD of two biological samples (bulked basal parts of 4 microshoots) and two technical repetitions. Samples were collected before IBA-treatment (0 h) and after IBA-treatment at 2 h and 14 days.

5.5. Discussion

Adventitious organogenesis, such as root induction at microshoots, can be seen as a reaction of shoot cells upon stress (Arnholdt-Schmitt et al. 2006b, Macedo et al. 2009) comparable to the process of stress-induced somatic embryogenesis (Zavattieri et al.

2010). Acclimation to stress can be expected to be accompanied by metabolic re-adjustment. However, the exact nature of stress-induced metabolic re-adjustment is poorly understood and represents an area of considerable research interest (Lehmann et al. 2009, Vanlerberghe et al. 2009). The present paper adds knowledge to recent state-of-the-art insights by studying association between AOX activity and secondary metabolism during adventitious rooting in olive microshoots.

The present results show that IBA-induced adventitious rooting in olive microshoots can be significantly suppressed by the AOX inhibitor SHAM including the suppression of morphogenic fields differentiation. This observation points to a role of alternative respiration for the induction of adventitious roots. Induction of new cell programs covers different phases that starts from dedifferentiation and ends by the initiation of cell transforming and morphological events (Grieb et al. 1997, Zavattieri et al. 2010). However, SHAM treatment of olive microshoots failed to inhibit calli formation which is preceding the emergence of root primordia. A similar observation was reported by Hase (1987) in *Helianthus tuberosus*. Also in this species, SHAM affected rooting but did not inhibit calli formation. These results point to a role of AOX for root initiation in the later phase during induction rather than a role for dedifferentiation in the early phase of induction.

Alternative respiration is involved in diverse types of abiotic and biotic stresses. AOX has been proposed to play a master role in the organization of efficient acclimation of plants to changing environmental conditions (Arnholdt-Schmitt et al. 2006a, Vanlerberghe et al. 2009). Complex interaction between AOX activity and the signaling molecule H_2O_2 has been reported (Amirsadeghi et al. 2006, Gray et al. 2004, Popov et al. 1997, Umbach et al. 2005, Vanlerberghe and McIntosh 1996). It is of interest to note that in olive shoot cuttings H_2O_2-treatment could even substitute for the effect of added auxin as a root inducing agent (Macedo et al. 2009). In addition, SHAM has been found to interact with intercellular H_2O_2 in SHAM treated *Arabidopsis thaliana* roots, where H_2O_2 disappeared from the root hair zone but accumulated in the cell walls of the meristematic region. In these experiments root length was significantly reduced (Dunand et al. 2007). Hilal et al. (1998) have reported on the

localization of AOX in meristem and xylem tissues in developing soybean roots and highlighted a link of AOX to xylem development under salt stress.

Involvement of lignin and phenolic acids in adaptive plant growth regulation, differentiation and organogenesis has been described by several groups (Fu et al. 2011, Ozyigit et al. 2007, Sircar et al. 2012). In the present olive system, reduction of root numbers in microshoots by simultaneous IBA and SHAM application was accompanied by down-regulation of the synthesis of secondary plant products from phenylpropanoid and lignin metabolism.

Phenolic acids have been suggested to play an important role in co-operation with phytohormones in altering cell wall composition and, thus, affect differentiation and morphogenesis (Cvikrová et al. 1998, 2003; Hartman et al. 1996). It is probable that phenolic acids form a complex with indole acetic acid (IAA), a key factor for root differentiation and development (Balakrishnamurthy and Rao 1988, Fu et al. 2011). Furthermore, phenylpropanoid derivatives have been reported to mimic the effects of cytokinins in regulating cell division and differentiation (Tamagnone et al. 1998). Stimulatory effects of phenolic acids and flavonoids on adventitious rooting have been observed previously in many plant species (Curir et al. 1992, Fu et al. 2011). Moreover, anthocyanin and flavonoid biosyntheses, which originate from phenylpropanoid pathway intermediates, correspond to AOX activity as has been demonstrated in transgenic *Arabidopsis* (Fiorani et al. 2005).

In our study on the transcription of two individual *AOX* genes *OeAOX1a* displayed higher transcript level than *OeAOX2*. However, both *OeAOX1a* and *OeAOX2* showed stable transcript accumulation at all selected time points when primers from ORF region were used. Lehmann et al. (2009) have pointed to a general lack of correlation between metabolites and involved transcripts. Interestingly, differential transcript accumulation of *OeAOX2* was observed when the reverse primer had been designed in the 3'-UTR. High level of *OeAOX2* transcript was observed at day 14 of culture after IBA-treatment which is the initiation point of root morphogenic field formation. Furthermore, this result is in agreement with previous results where differential transcript accumulation was found for shoot cuttings of cv. Cobrançosa (Macedo et al. 2009). In the 3'-UTRs of *OeAOX2* transcripts of cv. Galega vulgar

sequence and length variability were identified (Santos Macedo et al. 2009). Thus, the results indicate the possibility for differential regulation of *OeAOX2* during adventitious rooting through different classes of transcripts from the same gene (Polidoros et al. 2009). The functional meaning of such type of post-transcriptional regulation of *AOX* genes in olive rooting will be explored in future experiments.

In conclusion, the data support the general hypothesis that *AOX* genes can play a key role in the process of adaptive cell-reprogramming. A non-inhibitory effect of SHAM on calli formation but inhibition of rooting makes olive adventitious rooting in microshoots a highly interesting experimental system for future research. It will enable the separation of different stages in re-programming, such as re-determination and initiation of morphogenetic changes (Grieb et al. 1997, Zavattieri et al. 2010). Since phenylpropanoid and lignin accumulation was suppressed by SHAM treatment, it can be postulated that AOX control of the process of root formation associates with alteration in phenylpropanoid and lignin metabolism which in turn interact with meristematic growth (Sieger et al. 2005). A role of 3'-UTR variability in posttranscriptional AOX gene regulation is indicated and needs further clarification.

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Chapter 6 General Discussion

The most important cultivars used in Portugal are ‘Galega Vulgar’, ‘Carrasquenha’, ‘Cordovil’, ‘Cobrançosa’ and ‘Verdeal’ (Gouveia 1995, Bartolini et al. 1998). Portuguese olive cultivars used most in the olive oil industry are those involved in the production of olive oils with Protected Denomination Origins (DOP) (Bartolini et al. 1998, Gouveia et al. 2002). Of these cultivars, ‘Galega vulgar’ is the predominant one and accounts for 80% of the olive plantations in Portugal (Gemas et al. 2002). The olive oil produced is high quality due to the high content of tocopherols and it is highly prized by consumers. Due to the role of Portugal as a producer and an exporter of olive oil this cultivar has particular relevance in terms of global markets.

The traditional methods used for olive propagation since ancient times are rooting of cuttings and grafting of stem segments onto rootstocks (Lambardi et al 2013). The use of cuttings is a rapid, simple method of propagation for maintaining genetic uniformity and they come into bearing earlier than those raised from seeds (Ullah et al 2012). The semi-hardwood propagation technique was widely used from 1950 to 1960 and is now the most common method of olive propagation (Peixe et al 2013).

The most widely used method for grafting in nurseries for obtaining grafted plants is the method of fork placement at the top of the plant from seeds. This method has also been tested for grafting of self-rooted plants, to take advantage of using clones of rootstocks. However, because of the very high cost involved, this method has not been widely adopted. Grafting of seed plants dates back to ancient times, but in general the problems identified are always the same: difficulties encountered in seed germination and the time required to produce a plant capable of being grafted (Peixe et al 2013).

The cloning of 'Galega vulgar' using processes of vegetative propagation has been hindered by the low rate of rooting of semi-hardwood cuttings (Peixe et al. 2007a). The AR process has been subject of numerous studies aiming to provide a better

understanding of the process and develop methods of improvement. However, for cv. Galega vulgar, there is little information available and techniques to effectively improve the rate of rooting have not been developed despite the efforts made (Jacob and Matthias 2012). The findings of the national project entitled 'Development of integrated strategies for the recuperation of the cv. Galega vulgar as a major olive cultivar for Portuguese olive oil production' (2003-2007) reported that out of the 64 clones studied from this cultivar 25% did not present any root and among the remaining 75% the best rooting result was 34% and the rooting average of all the clones was just 4%.

In view of the obstacles in the way of achieving the goal of improved rooting capacities of cv. Galega vulgar and other badly rooting olive tree germplasms in the most important system for tree propagation in commercial terms, an alternative strategy via breeding with FM assistance is proposed as part of the present research study. This thesis follows an accepted hypothesis-driven, complex research approach for functional marker candidate identification mainly based on two papers: the first of these describes the theoretical strategy for molecular marker identification in target tissues for selected breeding traits (Arnholdt-Schmitt 2005); the second justifies the selection of AOX as a marker candidate source for efficient stress behavior which also includes a common strategy for experimentation (Arnholdt-Schmitt et al. 2006a). The two experimentation strategies were combined, adjusted for the research approach of the thesis and presented at an early stage in its writing to the scientific community for debate (Arnholdt-Schmitt et al. 2006b, Macedo et al. 2008). The general theoretical approaches were recently confirmed and updated in accordance with publications by Arnholdt-Schmitt 2009, Polidoros et al. 2009 and Cardoso and Arnholdt-Schmitt 2013.

AR can be described as a process of cell-reprogramming, in which cells from the stem gain the capacity to redifferentiate and subsequently respond by developing a new organ. As described in Chapter 1, AR can be interpreted as a three-phase process. The first phase is characterized by molecular and biochemical modifications related to the cell response to abiotic or/and biotic stresses such as wounding or auxin exposure. Several reports describe the involvement of AOX in biotic and abiotic stress responses

(McDonald and Vanlerberghe 2006, Plaxton and Podestá 2006). It is also well known that plants generate ROS in response to wounding and consequently cell death (Orozco-Cárdenas et al. 2001), and the involvement of AOX in the control of ROS content in plant cells diminishing oxidative stress and maintaining cell homeostasis is well documented (Vanlerberghe 2013). Additionally, AOX is known to be involved in controlling retrograde communication based on the networking between mitochondria and nucleus at cell level (Clifton et al. 2006, Vanlerberghe 2013).

Physiological studies performed as part of the present thesis demonstrated the involvement of AOX in AR. The use of the AOX inhibitor SHAM and AOX inducers (H_2O_2 and pyruvate) were directly correlated with a decrease and an increase in AR ratios, respectively. The trial regarding AOX expression carried out as part of this study showed that *OeAOX2* was highly expressed 10 min after shoot cutting from cv. Cobrançosa and microshoots from cv. Galega vulgar before IBA treatment, indicating AOX involvement in the first phase of AR.

The second phase is known as initiation and is characterized by the new entry into the cell cycle of former quiescent cells and subsequent root primordial organization (Li et al. 2009). Root primordial in IBA-induced olive shoot cuttings becomes visible in the commercial system from the 14^{th} day after auxin treatment. The anatomical study performed in microshoots revealed that the development of morphogenetic fields in olive microshoots could be identified in 14 days after explant inoculation under growth conditions. In the trials where SHAM was used, it inhibited the development of new morphogenic fields, indicating the involvement of AOX in the initiation of root formation. In the trial of AOX expression in this study, a high level of *OeAOX2* transcript was observed at day 14 of culture after IBA treatment from shoot cuttings from cv. Cobrançosa and in microshoots from cv. Galega vulgar, showing AOX involvement also in the second phase of AR.

The findings presented on secondary metabolite quantification showed that SHAM application negatively influences phenylpropanoid metabolism, decreasing the biosynthesis of flavonoids, total phenolic compounds and lignin, all of these important

substances involved in cell differentiation (Cvikrova et al. 2003, Palama et al. 2010) and demonstrated to be associated with AR (Fu et al. 2011).

The initiation phase is also characterized by coordination of cell repair, DNA replication and cell elongation processes necessary for AR, thus requiring a considerable input of energy and structural carbohydrates (Ahkami et al. 2008, Husen 2008). Several authors have suggested that AOX plays a key role in regulating cell-reprogramming by ameliorating metabolic transitions associated with the cellular redox state and the flexible carbon balance (Arnholdt-Schmitt et al. 2006a, Rasmusson et al. 2009).

The third phase of AR is characterized by growth and emergence of adventitious roots (Li et al. 2009) On the basis of the histological event chronology during AR in microshoots it was possible to deduce the negative effect of SHAM on root primordia formation in cv. Galega vulgar microshoots at day 19 of IBA+SHAM application, suggesting the involvement of AOX in adventitious root development.

For FM development, it is not necessary to understand the details of the involvement of the target gene for FM sources in metabolic networking. What is important is the identification of a target gene candidate and the follow-up association between any polymorphisms at DNA level to the final breeding trait, in this case efficient AR. Therefore, after having demonstrated the general involvement of AOX in AR, it made sense to search for AOX gene polymorphisms that could be associated with AR efficiency (trait of interest). The isolation and characterization of the *AOX* gene family in olive was required for genomics and transcriptomics approaches. In addition, as occurs with other eudicot species, genes belonging to both *AOX*-subfamilies were identified: three genes belong to the *AOX1*-subfamily (*OeAOX1a*, *OAOX1b* and *OeAOX1c*), and one to the *AOX2*-subfamily (*OeAOX2*). The potential involvement of *AOX2* genes in plant development and at the same time in stress reactions (Clifton et al. 2005, Costa et al. 2007) led to the decision to select *OeAOX2* as a starting candidate for this study in order to search for sequence variability. The comparison of the partial *OeAOX2* gene sequences among cv. Galega vulgar and the easy-to-root cvs. Cobrançosa and Picual revealed the existence of sequence variability, the 3'-UTR being

the most important source of polymorphisms (5.7% of variability), followed by codifying sequences (3.4%) and intron 3 (2%).

SNPs and InDels contributed to the identified sequence variability in *AOX2* in cvs. 'Cobrançosa' and 'Picual'. The existence of a non-synonymous SNP is related to a nonsense mutation and consequently to the putative synthesis of a truncated protein. Several examples are known, in which the occurrence of this kind of natural mutation is associated with a loss-of-protein function, which links to a change in the phenotype. Such a mutation can be used also for application in breeding for FM development as was the case for the *GS3*, related to rice grain size, and *OVATE*, related to tomato fruit shape (Liu et al. 2002, Fan et al. 2009).

Besides sequence polymorphisms, 3'-UTR length variability was observed in the transcripts of two A*OX* genes, *OeAOX1a* and *OeAOX2*. This type of transcript variability may be related to alternative polyadenylation. In recent years, the alternative polyadenylation has been suggested as playing an important regulatory role during eukaryotic gene expression. Over 50% of studied plant genes possess multiple cleavage-polyadenylation sites (Xing and Li 2011).

In silico analyses suggest the existence of putative binding sites for miRNAs in the 3'-UTR of both genes. Interestingly, one of these sites, identified in *OeAOX2*, is homologous to the ppt-miR1212 localized in the 3'-UTR of the *A. thaliana* B-box zinc finger protein gene (Talmor-Neiman et al. 2006). Zinc fingers have been implicated in various important regulatory processes. Some of these proteins are implicated in plant responses to abiotic stresses. For example, in petunia, there is a gene codifying this type of protein, which responds to various stress treatments such as drought, cold, salinity and wounding (van der Krol et al. 1999), and in *Arabidopsis* there is another gene involved in drought tolerance (Sugano et al. 2003, Sakamoto et al. 2004). Further research is required in order to validate the results achieved for olive and highlight the association between gDNA variability to functionality in the target trait of efficient AR. Zinc finger domains in AOX genes may also provide an additional opportunity for establishing gene replacement strategies in order to target gene activities through the

induction of double-strand breaks [for further information on this technology see Pater and Hooykaas (2013)].

6.1. Conclusions and Perspectives

The studies performed in this thesis provide an important contribution towards achieving advances functional marker research with a view to molecular breeding on better rooting olive genotypes. In particular, this study contributes through:

i) the isolation and sequence characterization of the *AOX* gene family in olive;
ii) the establishment of a link between olive AR and AOX in a commercially relevant propagation system;
iii) the adaptation of an established *in vitro* microshoot culture system in order to facilitate the study of the question of an association between AOX and efficient AR and to screen AOX gene polymorphisms for functionality in rooting under defined conditions;
iv) the identification of target tissues for AR in olive microshoots of the poorly rooting cv. Galega vulgar and studying the involvement of secondary metabolism pathways (lignin);
v) highlighting the differential *AOX2* expression based on 3'-UTR length variability during the induction of secondary metabolism pathways related to AR;
vi) the identification of *AOX2* sequence variability and 3'-UTR length variation, which can now be used in future studies on FM development. Variability in the 3'-UTR was also discovered for *OeAOX1a*. A relationship with polymorphic differences at gDNA and root induction remains to be explored.

There is now a vast body of references available in the scientific literature which confirm that AOX is regarded as a main player in environmental- and developmental-related plant responses (Vanlerberghe 2013). AOX seems to be early responsive to external and internal changes in environmental conditions and relates to homeostasis and adaptation in metabolism and physiology. At the end of the thesis laboratory work, preliminary findings were obtained that indicate an early

increase in *AOX2* gene expression during the first hours of IBA treatment for root induction in the good rooting cultivar 'Cobrançosa' but not for the bad rooting cv. Galega vulgar under commercially important vegetative propagation conditions. These data are not included in this thesis, since they still require careful validation. However, thesis work is being continued by the research group linked to the Competence Focus AGRO-BIOTECH-AOX on 'AOX Research and Functional Marker Development'. The author of this thesis is involved in this effort. During the final stages of the thesis, the available *in vitro* system was used for further studies on the role of *AOX* genes in the rooting process and will finally serve for the screening for important polymorphic gene sequences as candidate functional markers. New findings on expression analyses confirm up-regulation in transcript accumulation of all AOX genes except AOX1b in the early induction phase with pronounced effects for AOX1a (Noceda et al. unpublished). Also, differential expression of *AOX2* genes with differing length in the 3'-UTR was confirmed by these studies, and the significance of this observation is being explored. The next steps will be further validation and screening of polymorphic *AOX* genes in relation to the observed early up-regulation during the induction phase of rooting in the *in vitro* system. Additionally, the research group has taken on new projects enabling the installation of new, unique technologies for measuring the effect of *AOX* polymorphisms. This includes measuring metabolic effects through oxygen consumption (measuring AOX capacities) and calorespirometry (Nogales et al. 2013, Hansen et al. 2009) as well as measuring AOX protein activities (new equipment is being designed as part of a recently-approved FCT project on AOX polymorphism screening through upgrading commercially-available instruments with help of an American Thermodynamics scientist, Lee Hansen, a consultant and research technology trainer at the EU Marie Curie Chair (see www.eu_chair.uevora.pt/communications).

Furthermore, the complete sequencing for all identified *OeAOX* genes should be performed, including *OeAOX1b, OeAOX2* and the validation of the *OeAOX1a* sequence (exon 1 seems to be short; 5'end sequences need to be completed). Additional information will also come from the isolation of the promoter sequences

and polymorphisms of *AOX* genes, which have been validated as candidate genes for FM development.

In order to understand the general meaning of identified functional marker candidates in a diversity of genetic backgrounds, large scale analyses should be extended to 'Galega vulgar' variants and other olive genotypes that are known to possess differences at the phenotype level for AR capacities in the commercial propagation system.

Agreements have been made for future collaboration with a range of research groups working on the genetics of olive material in Tunisia (official cooperation has been established, and contact made with a large olive producer) and Italy (informal collaboration agreed on) to map interesting *AOX* gene sequences in larger mapping populations that also contain the rooting phenotypes as mapped traits and also to perform association studies.

Future studies will also involve screening for *AOX*-related structural and epigenetic genome organization and regulative gene networking in order to enable more sophisticated FM identification strategies with a complex view on multifactorial contexts, focused on growth and development regulation in the applied, commercially relevant propagation systems.

The research carried out on this thesis is part of a broader Competence Focus on 'AOX Research and Functional Marker Development', which is also linked to several industrial pilot projects across a range of different organisms and applied systems. Thus, the findings of this thesis will be added to the overall body of knowledge that focuses and supports advances in the state of the art and also contribute in the future to ongoing projects. The most important findings are related to the relevance of the 3'-UTR region as a source for FM development, the early responsiveness of *AOX* genes in relation to selective plant plasticity (see induction of callus and rooting) and its potential link to the secondary metabolism in organogenesis, namely AR, and also potentially to related events such as the induction of somatic embryogenesis (see also studies on carrot root hairs in Sircar et al. 2012 and Frederico et al. 2009a and b). This thesis has also fostered the development of a new line of research for the hosting Chair and Competence Focus, which is currently

being presented at an international congress (Ragonezi et al. 2013, unpublished communication).

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