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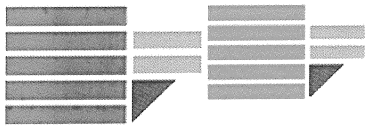

PhD in Plant Biology

PhD School "Life Sciences" XXV Cycle

In vitro **propagation of** ***Olea europaea*** **L. subsp.** ***europaea*** **var.** ***sylvestris*** **and molecular basis of its tolerance to stressful conditions**

Scientific disciplinary sector – 05/A1

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ABBREVIATIONS

PPM = Plant Preservative Mixture

OR = Olive Rugini

MS = Murashige and Skoog

ORZ = Olive Rugini Zeatina

MSZ = Murashige and Skoog Zeatina

Smc_s = Seedling microcuttings

Stc_s = Stem cuttings

Asc = axillary shoot cuttings

IBA = Indol-3-butyric acid

OesARF = Olea europaea L. subsp. europaea var. sylvestris AUXIN RESPONSE FACTOR

OesH3 = Olea europaea L. subsp. europaea var. sylvestris HISTONE 3

OesDHN = Olea europaea L. subsp. europaea var. sylvestris DEHYDRIN

OesH2b = Olea europaea subsp. europeae var. sylvestris Histone2b

qRT-PCR = Quantitative real-time PCR

Research aim

Olea europaea L. subsp. *europaea var. sylvestris* (Hoffmg *et* Link) is a widespread component of evergreen plant formation, commonly named "*Macchia*" in the Italian language, that extends along the coastal and subcoastal areas in the Mediterranean. "*Macchia*" consists predominantly of sclerophyllous shrub formations suitable to tolerate the arid conditions that mark the Mediterranean region. It is characterized by a high degree of biodiversity and is rich in endemic species, many of which are source of valuable products such as honey, liqueurs, fruits, herbs and numerous medicinal substances (Baratta *et* Barbera, 1981).

In Italy, the Mediterranean "*Macchia*" covers most of the coastal areas along peninsula and islands, although it is able to extend into slopes of the Apennines mountains, characterized by shallow soils subjected to rapid drainage. In this landscape, "*Macchia*" formation plays a major role in protecting soil from erosion as well as in ensuring an suitable hydrogeological structure. Notably, most of "*Macchia*" biodiversity is linked to Italian region.

Currently, the ongoing global climate change and the un-sustainable exploitation of land led to a worldwide increase of desertification threat, related both to the reduction of land water and to the depletion of natural resources. An evidence of this environmental threat is provided by the degradation and regression processes that are affecting, since the last 50 years, "*Macchia*" formation in the entire Mediterranean basin. Indeed, due to the above mentioned causes, first among all to the constant human pressure, in various countries, and particularly in Italy, "*Macchia*" formation appears highly fragmented and replaced, in most cases, by infrastructures, arid fields, eroded soils and bare rock. All these conditions strongly contribute to hydrogeological unsettlement impending on large parts of Italian coast. In this context, a recovery of the coastal landscape through an efficient and rapid restoration of plant formations in the degraded areas represents an urgent and challenging task.

Certainly, for a successful re-implantation of *"Macchia"* vegetation it is essential to use endemic species, which are already adapted to survive under ecologically unfavorable conditions. So far, propagation potentiality of most

"*Macchia*" species has been poorly explored. Moreover, despite a precise botanical characterization of these species, cyto-physiological and molecular bases underlying their tolerance to hard environmental conditions (i.e. low water content, high irradiance, high temperature, nutrient scarcity) are not yet fully understood. As a consequence, in the approaches that have been carried out for restoring degraded landscapes, a limited number of species have been used, usually corresponding to the most easy to keep in the nursery. In turn, this resulted into an impoverishment of biodiversity, a simplification of ecosystems and landscapes and, at the end, into a risk of approach failure due to the use of unsuitable genotypes. On this basis, it becomes important, on one hand, to improve technology for vegetative propagation and, on the other hand, to expand molecular knowledge on "*Macchia*" autochthonous species, which so far have not been conventionally used for landscape restoration, while adapting easily to "extreme" environments, such as sites next to sandy, rocky and clay coasts.

The present PhD project is part of a research line addressed to respond to above expressed aims. As previously mentioned, the attention has been paid on *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Hoffmg *et* Link), commonly named *Oleaster,* which represents the wild form of olive. *Oleaster* is largely prevalent in the Mediterranean vegetation due to a high photosynthetic efficiency and drought tolerance, related to its tap-root system that allows a deep exploration of the soil. (Mulas, 2009). So far, *Oleaster* is only used as rootstock and pollen donor for many cultivated varieties of olive, while it could be successfully applied for the restoration of eroded soils. Indeed, in addition to the above mentioned characteristics, *Oleaster* easily implants itself both on sandy soils and rocks (Bacchetta *et al.,* 2003). Unexpectedly, despite these features, wild olive aroused scarce interest among scientific community involved in research on olive species.

In this context, the present research project pursued a double objective:

1. to set up an efficient procedure for *Oleaster* micropropagation and, at the same time, to identify early molecular markers of adventitious organogenesis;
2. to extend molecular knowledge on the tolerance of *Oleaster* plants to stressful conditions.

CHAPTER 1

The Mediterranean Vegetation in the World

1.1 - Distribution and communities types

The Mediterranean vegetation commonly named "*Macchia*" is one of vegetation types present in the biome which colonizes world's lands characterized by Mediterranean climate. (*Fig.1.1*). The typical structures of the Mediterranean biome have been analyzed by many authors (Pignatti, 1998 and reference herewith cited). Briefly, it includes the following major plant communities: shrublands, forests, woodlands, savannas and grasslands; "mosaic habitat" landscapes are also common. Mediterranean "*Macchia*" belongs to "shrublands" type, formed by dense thickets of evergreen sclerophyllous shrubs and small trees which are called, besides *macchia* (Italy), *chaparral* (California and southern Portugal), *matorral* (Chile and southern Spain), *maquis* (France and elsewhere around the Mediterranean), *fynbos* (South Africa) or *kwongan* (Southwest Australia).

The Mediterranean climate is considered as a transition point between the temperate and the tropical-arid climates which evolved during the Tertiary from hot and humid conditions present after global climate settling (Di Castri *et* Mooney, 1973). In the world, the Mediterranean climate is present on the west coast of continents at the mid-latitudes (i.e. between 30° and 45° latitude in both hemispheres) (Bussotti *et* Schirone, 2001) and is characterized by hot summers, winters mild to cool, rainfall concentrated in winter, high variability in annual precipitation, a long period of summer drought, absence of temperature fluctuations typical of continental climates. The Mediterranean climate zones includes: the Mediterranean Basin; the Chilean Matorral; the California chaparral and woodlands eco-region of California and the Baja California Peninsula; the Cape Province-Western Cape of South Africa and the Southwest Australia corner area (*Fig.1.1*) (Specht, 1969; Valiente-Bonuet *et al.,* 1998). These five areas cover only 2% of the planet's land surface but are home to more than 20% of species on the planet, representing an important reservoir of biodiversity.

Figure 1.1. Global distribution of areas with Mediterranean vegetation

1.2 – Eco-physiological Aspects

The strategies developed by plants to cope with stressful conditions can be classified into two broad categories: '*resistance*' and '*tolerance'*. The first consists in the set of mechanisms that plant activates to prevent the stress; the second consists in the set of mechanisms that enable the plant to carry out its vital functions even under stressful conditions.

For Mediterranean species stressful conditions comprises drought, high irradiance and temperature which occur during summer. The resistance strategies developed by Mediterranean species to survive to these conditions include: leaf fall, reduction of vegetative system, reduction of transpiration through the stomata closure, a very extensive and deep root system and in some cases a real state of summer 'rest'. Concerning the enhanced root development, it allows the plant to absorb water from the soil even under severe drought. However, in these conditions plants are subject to very high waste of starch reserves in order to can carry out photosynthesis in presence of strong negative water potentials in the leaves.

Among tolerance-related strategies, various mechanisms counteracting dehydration have to be counted. One of the most investigated is the presence of small sclerophyllous leaves, which are characterized by epidermal cells exhibiting thickened cell walls, with thick cuticle, and a very dense mesophyll formed by several layers of palisade tissue. These features protect the leaf from excessive

transpiration but, at the same time, reduce its photosynthetic efficiency and growth rate. In addition, leaf cuticle is often impregnated with substances which play protective role but have a very high metabolic cost, thus taking away a lot of energy to the growth. It is generally accepted that sclerophyllous leaves represent an adaptive response of Mediterranean plants but sclerophyllous species are not exclusive to these environments, being widely spread in hot and humid regions like the Macaronesian (Canary Islands). Therefore, it has been suggested that the "habitus" of Mediterranean sclerophyllous species is derived from laurophyllica anatomical structure typical of species evolved in wetlands and only later naturalized in arid climates (De Lillis, 1991).

In conclusion, from a morphological point of view the Mediterranean species may resort to a broad spectrum of possibilities to complete their life cycle (Bussotti *et* Schirone, 2001)

1.3 - Mediterranean "*Macchia"*

The "*Macchia"* is a shrubland biome of the Mediterranean region, tipically consisting of densily growing evergreen shrubs, with a floristic composition similar to that of evergreen forests, although it lacks of arboreal individuals. It may be the result of a combination of hard climatic and edaphic factors, that maintain the *cenosis* in a condition of *paraclimax,* preventing the evolution toward forestry structure (primary "*Macchia"*) or it may arise from evergreen forest as a result of human disturbance actions such as repeated fire, grazing or frequent cuts (secondary "*Macchia"*). Currently, much of the current "*Macchia"* is considered a secondary formation compared to the original Mediterranean evergreen forest which regressed due to different factors acting at significantly different times and exerting a different impact.

"*Macchia"* is found throughout the Mediterranean Basin including most of Italy coast, southern France, southern Portugal, Lebanon**,** Sardinia, Corsica, and elsewhere. Among species it includes: *Erica arborea* L.*; Quercus ilex* L.*; Arbutus unedo* L.*; Salvia officinalis; Juniperus communis* L.*; Euphorbia dendroides* L.*; Olea europaea* L. *var. sylvestris; Myrtus communis* L.

It should be specified that different species, typical of "*Macchia"*, that generally develop as shrubs, actually take an arboreous habitus if disruptive actions were to stop. It 's the case, for example, of *Quercus coccifera* L.*, Juniperus communis* L.*, or Phillyrea latifolia* L. which under favorable conditions gave rise to forests, as in some parts of Sardinia and Corsica (Bussotti *et* Schirone, 2001).

Depending on height, density and species composition, Mediterranean "*Macchia"* can be classified under several categories. In Italy, we can distinguish the following main formations:

- *Nerium oleander* L. riparian formations, present along rivers and streams where the temporary period of dryness is very long;
- *Quercus coccifera* L.*,* formation, spread mainly in Puglia and Sicily;
- *Juniperus communis* L., formation , present on the coastal consolidated dunes, especially in Sicily and Sardinia;
- *Olea europaea* L. var. *sylvestris* and *Pistacia lentiscus* L. formation, that is the most common formation of the coast and include very thermophilous species;
- *Heather* low scrub, *Cistus spp.* (*C. incanus, C. monspeliensis, C. salvifoliu*s) and *Lavandula stoechas* L. formation, which represents an extreme form of degradation before the "*garrigue"*; it develops on acid and nutrient-poor soils, frequently crossed by fire.

1.4 - Depauperation and regression of "*Macchia*": an ecological threat

As already mentioned, several disturbance factors, each other strongly correlated, impend on "*Macchia*" association. These factors can be summarized as follows: fire, overgrazing, climate change and over-exploitation of land due to an ever-increasing human population, urban and industrial development, as well as tourism-related activities These latter especially impacted on the coastal areas where numerous and extensive infrastructures (roads, railways, pipelines, houses, etc.) took place. In addition to the disturbance factors related to human activities, it must be considered that "*Macchia"* is naturally subjected to environmental factors, such as high temperatures and drought, which they selves feature as stressors towards vegetation.

Separately and together, all these factors led to a less complex plant association with a reduced number of species, resulting into serious threat for biodiversity (Davis *et* Richardson, 1995). Moreover, the current global changes (i.e. increase of temperature and concentration of tropospheric O_3, CO_2 and other greenhouse gases) also have a great potential for altering the balance that allows "*Macchia"* association to survive (Rossi *et* Duce, 1999). The Intergovernmental Working Group on Climate Change (IPCC, 1995, 2001) reported an average temperature increase, in the last century, of about 0.8°C in large part of Europe; such value is significantly higher than average temperature increase globally registered during the same period (about 0.6°C**).** The trend of climate to become warmer and less rainy has been confirmed also for Italy (Brunetti *et al.,* 2004). These trends are to be taken into special consideration because the distribution of "*Macchia"*, like for all forest ecosystems, strongly depends on climate. An increase in temperature, which would prevent adaptive evolution of species to new conditions, could in fact cause a shift of the phytoclimatic band to more northern latitudes and higher altitudes (Zinoni *et* Duce, 2003).

Currently, the synergic effect of above mentioned events on "*Macchia"* vulnerability plus the difficulty in planning a sustainable use of natural resources, are causing a significant reduction of the area covered by "*Macchia"* ecosystem, thus reducing biological resources and contributing to deleterious processes of soil degradation and desertification. Indeed, the dense and extensive plant cover formed by "*Macchia"*, if preserved, can effectively prevents soil erosion caused by rain and wind through plant root system which contributes to maintain soil stability. Therefore, the degradation of plant formation strongly impacts also on landscape damage.

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CHAPTER 2

STUDY MODEL*: OLEA EUROPAEA* L.

subsp *europaea* var. *sylvestris*

2.1 - Botanical Classification and origin of olive (*Olea europaea* L.) complex

The taxonomic position of olive (*Olea europaea* L.) has recently been revised at the light of data emerging from molecular approaches (Green, 2002). According to this revision, olive (*Olea europaea* L.) tree belongs to the family of *Oleaceae,* which includes the genus *Olea*, *Fraxinus*, *Forsythia*, *Forestiera*, *Ligustrum* and *Syringa*.

The *Olea europaea* species comprises six subspecies, defined on the basis of morphological characters and geographical distribution. They are listed below:

Olea europaea subsp. *cerasiformis* present on the island of Madeira;

Olea europaea subsp. *cuspidata* spread from Iran to China;

Olea europaea subsp. *europaea*, the Euro-Mediterranean olive, which includes two botanical varieties: the variety *sativa* (olive of cultivation); and the variety *sylvestris* (wild olive) (*Fig. 2.1)*;

Olea europaea subsp. *guanchica* present in the Canary Islands;

Olea europaea subsp. *laperrinei* present in the region of the Sahara;

Olea europaea subsp. *maroccana* present in Marocco.

However, it must by underlined that very often taxa with different "geographic" distribution show morphological characteristics quite similar, thus making difficult the taxonomy of olive complex (Besnard *et al.,* 2001). This triggered an even increasing utilization of different molecular markers (Green, 2002). Likely, the above mentioned difficulties are related to the origin and evolution of different taxa within olive complex (Besnard *et al.,* 2001). It is assumed that olive progenitor originated in Africa during the Paleolithic and colonized Mediterranean area during the Pliocene (Besnard *et al.,* 2001). Probably, until the end of the last ice age, olive species was predominantly distributed in Africa but desertification led to

the subsequent isolation of Sahara populations belonging to subsp. *laperrinei*. Under these conditions, the Mediterranean should have been colonized by different forms, likely distinguishable through molecular features, and it is believed that only after the last glaciation the wild olive of the western Mediterranean underwent to a redistribution, reaching the coasts of Spain and France (Besnard *et al.,* 2001).

2.2 - *Olea europaea* subsp. *europaea:* morphology, phenology and reproductive biology

Olea europaea L. subsp. *europaea*, includes two varieties: var. *sativa* and var. *sylvestris*; this latter is commonly named *Oleaster* and represents the wild form of olive (Fig. 2.1). Currently, it is believed that the wild olive, *Olea europaea* subsp. *europaea* var. *sylvestris*, represents the original population of *Olea europaea* subsp. *europaea* growing in postglacial Mediterranean (Zohary *et* Splegel Roy, 1975; Zohary, 1994).

Olea europaea L. subsp. *europaea* features as a tree (var. *sativa*) or a shrub (var. *sylvestris*) whose age is believed to exceed in many cases 1000 years old. The trunk appears often twisted and in the monumental specimens it can reach considerable size. The bark, gray colored, is more or less smooth in young trees and becomes wrinkled in adult ones. The leaves are opposite, elliptic-lanceolate in cultivated form and rounded in wild form, leathery, with smooth margin and green and glabrous upper surface. The flowers, white and very numerous, are born on axillary inflorescence, named olive blossom. The corolla consists of four petals (2-4 mm long) with rounded apex , developed more in length than width; there are two stamens for flower; the stigma is bifid and the ovary exhibits four niches. The fruit is an oval drupe whose mesocarp springs initially green then blackish brown at maturity; the endocarp is hard and woody and includes one, rarely two seeds (Mulas, 2005).

Seed germination occurs between mid-February and mid-March, depending on the geographic area. Plant growth is high from March to June while it is almost absent in the summer because of drought. Flowering starts in May and lasts until early June. The flowers are formed on the branches of the previous year. Olive

reproduces sexually and pollination is anemophilous; and dissemination is assured by gravity or birds (thrushes, blackbirds) that feed of mature drupes (Alcántara *et al.,* 2000).

The main morphological differences between cultivated and wild varieties deal with: fruit size (fruit average weight varies between 1 and 12 g while rarely reaches 0.5 g in cultivated and wild form, respectively); fat yield in fruits (higher in cultivated olive); leaf size and shape (much smaller and rounded in the wild form, larger and elliptical-lanceolate in cultivated olive).

The two varieties are fully interfertile and exhibit the same chromosome number of (2n = 46) (Zohary *et* Spiegel-Roy, 1975; Besnard *et* Bervillé, 2000; Contento *et al*., 2002). Triploid and tetraploid conditions as well as a polysomy (2n = 55) have been occasionally found (Breviglieri *et al*., 1954). Both varieties show a good grafting affinity (Deidda *et* Mulas, 1998). Accordingly, *Oleaster* is used as rootstock and pollen donor for many olive cultivars.

2.3 - Habitat and Climatic Requirements

Olive plant grows at altitudes ranging from 0 to 800 m above sea level, on all types of soil although olive prefers clay and well-drained soils which avoid water stagnation. For an optimal vegetative growth olive plant requires fertilization with high nitrogen content while during the fruiting an increased potassium level is required in order to accumulate carbohydrates. However, due to its wide root system olive plant is able to survive even on mineral-poor soils (Cimato *et* Fiorino, 1985). Although olive plant may resist to low temperatures, between 0 e -8° C, the optimum temperature for growth is between 22 and 28 ° C.

Despite its adaptative flexibility,, growth and productivity of olive plant as well as quality of its products are deeply influenced by climatic conditions, first of all by temperature and water availability (Alfei, 2009). In addition, plant physiological performance is also influenced by other environmental factors such as wind, excessive rainfall and high humidity (Mulas *et al.,* 2003).

The wild olive form requires an intense light radiation for its vegetative growth (Tombesi *et* Cortechini, 1986) and exhibits traits of higher tolerance to

various environmental stresses compared to the cultivated one: it exhibits a high photosynthetic efficiency and drought tolerance, related to its tap-root system that allows a deep exploration of the soil (Mulas, 2009); it can survive on land with a wide pH range (from 5.8 to 9.0); it is extremely tolerant to salinity. According to these features it is widespread along the coasts and coastal areas (Cimato *et* Fiorino, 1985).

Figure 2.1: *Olea europaea* L. subsp. *europaea* var. *sylvestris*. Along Calabria's Tyrrhenian coastal area. From Cinzia Gagliardi.

2.4 – The Potentiality of *Oleaster* in landscape management and re-naturalization

From an ecological point of view, *Oleaster* exhibits many relevant features. First of all, due their prevalence within "*Macchia*" association, wild olive populations play a major role in protecting soil against erosion, by consolidating land through the wide root system. Moreover, on account of *Oleaster* resistance to wind and drought, they contributes to the survival of natural plant communities, while their ability to recover after a fire through new shoot formation assures a rapid rebuilding of the original vegetation cover (Mulas *et* Deidda, 1998).

It is clear that to be successful in re-naturalizing highly degraded lands or, simply, for ensuring some degree of soil cover, the species/varieties of plants, which have to be used, must be selected on the basis of several independent features such as: high attitude to reproduction by seed and/or shooting; high attitude to vegetative propagation; high stress tolerance (Mulas, 2005). Certainly, varieties that either possess a high germination rate or easily undergo to vegetative propagation can assure a successful colonization and full expression in the territory. On the other hand, shooting faculty and stress tolerance are intrinsically vital to the single plant that is facing a climatically hardy environment, difficult to became colonized and where a strong capacity to persist and resist to both climate and human pressure (grazing, fire, etc.) is required. For all these reasons wild olive appears largely apt to be used in reforestation and management of eroded areas of Mediterranean *"Macchia"*.

CHAPTER 3

RESEARCH WORK PLAN

On the basis of research double objectives, the work plan included two sections:

Section A. *In vitro* propagation of *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Hoffm. *et* Link): culture conditions

The work done in this section dealt with:

I. The set up of efficient protocols for seed germination:
II. The set up of optimal protocols for "*in vitro"* growth and rooting:
    (a) of microcuttings obtained from seedlings;
    (b) of microcuttings excised from field-grown plants;
III. The histological analysis of adventitious roots developed during "*in vitro"* microcutting propagation

Section B. *In vitro* propagation of *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Hoffm. *et* Link): molecular markers of adventitious root formation.

The work done in this section dealt with:

I. The analysis of expression level of the following genes, both involved in the induction phase of adventitious root formation.:

i) *OesARF* (*Olea europaea* L. subsp. *europaea* var. *sylvestris AUXIN RESPONSE FACTOR*), a member of ARF genes family;

ii) *OesH3* (*Olea europaea* L. subsp. *europaea* var. *sylvestris HISTONE 3*), a member of *HISTONE* gene family;

Such analysis was performed in microcuttings derived from seedlings, during the induction phase of rooting process.

Section C. Stress tolerance of *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Hoffm. *et* Link) plants: molecular aspects.

The work done in this section dealt with:

I. The analysis of expression level of *OesDHN* (*Olea europaea* L. subsp. *europaea* var. *sylvestris* DEHYDRIN), a member of SK_2-type subgroup of *DHN* gene family, known to be involved in many different stresses

Such analysis was performed in:

II.1 plants grown in the fields;

II.2 micro-propagated plantlets exposed to low temperatures;

In both B and C sections, we took advantage from a cDNA library of *Oleaster*, made available by Prof. M.B. Bitonti's research group.

In the following chapters, the work done in each section will be quoted separately by describing background, material and methods that were used and the obtained results. It will follow a general discussion of the results and the concluding remarks. I selected to follow this schedule in presenting the work I did in order to facilitate the reading to those interested only in some parts of the work.

CHAPTER 4

SECTION A- *In vitro* propagation of *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Hoffm. *et* Link): culture conditions.

4.1 BACKGROUND

4.1.1 - Seed Germination

In nature, seed germination is regulated by dormancy process (Acebedo *et al.,* 1997). Dormancy can be related to both endogenous and exogenous (light, temperature) factors; on the other hand endogenous dormancy can be imposed by both morphological and physiological features of seed and fruit. For example, in the endogenous dormancy of olive seed the woody endocarp results into a mechanical resistance to germination, while inhibitory substances, presumably localized in the seminal integuments and in the endosperm, may interfere with embryo germination even after removal of woody endocarp. In fact, experimental tests have shown that the nude embryo is not affected by dormancy and germinates quickly after a few days of *in vitro* culture, while the whole seed (embryo, endosperm, and seed coat) needs to be stimulated to germinate by treatments with ethylene and cytokinin (Rinaldi*,* 2000).

To overcome dormancy and promote germination the most common pretreatments used in nursery practice are: *Scarification* and various types of *Stratification. Scarification* consists of abrasion of seed outer integuments, especially those particularly hard that impose physical dormancy. *Scarification* can be performed by mechanical, physical or chemical (using acid or alkali) treatments which cause the loss of integrity of seed integuments, thus furthering water absorption, gas exchange, and consequently germination process (Suszka, 1978; Muller, 1992). *Stratification* is a process which consists in the layered arrangement of seeds in a soft and moist medium, generally consisting of peat, perlite, vermiculite or sand, which can be used individually or mixed together in various proportions.

The *stratification* can be carried out at controlled low temperatures (between +2 and +5 ° C) and humidity (in refrigerators, cold rooms, etc.) or at room low temperature and also outside (in boxes, in the ground, etc.). It is called *cold stratification* or *vernalization* and has the primary objective of removing endogenous physiological dormancy, but also of affecting seminal integuments thus accelerating water absorption. The *stratification* performed at around + 20 ° C is called, instead, *hot stratification* or *aestivation*: it mimics the effects of the summer on the biology of the seed that needs relatively high temperatures to complete the embryo development (Suszka *et al.,* 1994). Note that the term *'stratification',* without specifying whether *'hot'* or *'cold'*, refers to hot stratification*,* cold stratification being commonly defined as *vernalization*.

4.1.2 - Micropropagation

Micropropagation is a technique of agamic propagation and for many species it has become an alternative system with respect to other traditional techniques of propagation. The objective of this methodology is to obtain, in a short time and at low costs, a large number of seedlings that exhibit the same genotype and phenotype of mother plant. It was used for the first time for orchids (Morel, 1965) and at the moment is largely applied in the fields of agronomy, floriculture and forestry to obtain plants with previously selected physiological characteristics and production of merit.

Four fundamental phases, from I to IV (*Fig.4.1.1*), are required to make a successful micropropagation. These phases, beside describing the procedures used during micropropagation, mark the points at which culture conditions are to be changed (Miller *et* Murashige, 1976). Certainly, the requirements for the completion of each phase depends mainly on the plant material but also on the specific methods that are used.

Phase I: Explants Preparation

The objective of this phase is to place selected explants into culture, avoiding contamination and providing an environment that promotes shoot production (McCown, 1986). Depending on the type of explant, shoot formation may be

initiated from pre-existing meristems (apical and axillary buds), from adventitious meristems (direct organogenesis) that originate on excised organs (including cotyledons, leaf, shoot, bulb scale, flowers, stem) or from callus that develops at the cut surfaces of explants (indirect organogenesis). Usually 4–6 weeks are required to complete this stage and to generate explants that are ready to be moved to Phase II (Hartmann *et al.,* 2002).

Phase I (McCown, 1986) is also called '*stabilization'* phase. A culture is stabilized when explants produce a constant number of shoots after subculture (Hartmann *et al.,* 2002). Some woody plants may take up to12 months to complete this stage.

Phase II: Proliferation of in vitro explants

Phase II is characterized by repeated cycles of axillary bud proliferation from smaller shoots, individual shoots, apical nodal segments or cuttings excised from shoots obtained from primary explants and subsequent subcultures. In this phase, a culture medium containing high cytokinin levels is used in order to inactivate the apical dominance of terminal buds and to promote axillary bud development. Usually, subcultures are performed at intervals of 4 weeks. The number of subcultures which can be performed depends on the species or cultivar (Kurtz *et al.,* 1991). This phase represents one of the more expensive step of propagation process and, in average, lead to a 3-8 fold increase in the number of shoots.

Phase III: In vitro Rooting of Explants

The rooting phase is necessary and preparatory for transplanting the regenerated plantlets from *in vitro* to *ex vitro* conditions, first in controlled growth chamber, then in glasshouse and, later on, in the open field. Therefore, this phase includes not only rooting procedure, but also the conditioning of the plants in order to increase their potentiality to acclimatize and survive after transplanting. The induction of adventitious roots is achieved by using auxin and can be performed either *in vitro* or *ex vitro* (Ostroluck´a *et al.,* 2007; Gajdoˇsov´a, *et al.,* 2007). The main advantage of *ex vitro,* compared to *in vitro* rooting, is that in such way it is avoided root damage during transfer to soil. Moreover, the rate of root production is

often higher and root quality is optimized when rooting occurs *ex vitro* (Bonga *et* Von Aderkas, 1992; De Klerk *et al.,* 1997).

Phase *IV: Acclimatation*

During this phase the regenerated plantlets are transferred on the soil under natural environmental conditions (Hartmann *et al.,* 2002). Transplantation of *in vitro* derived plantlets to soil is often characterized by lower survival rates. Before transferring soil-rooted plants to their final environment, they must be acclimatized in a controlled growth chamber or in the glasshouse (Preece *et* Sutter, 1991;Rohr *et al.,* 2003). Plants transferred from *in vitro* to *ex vitro* conditions, undergo gradual modification of leaf anatomy and morphology: for example closing/opening mechanism of stomata, which are usually open in culture-grown plants, begins to function; plants also start to produce protective epicuticular wax layer over the surface of their leaves. Regenerated plants gradually become adapted to survival in their new environment (Donelly *et* Tisdall, 1993)

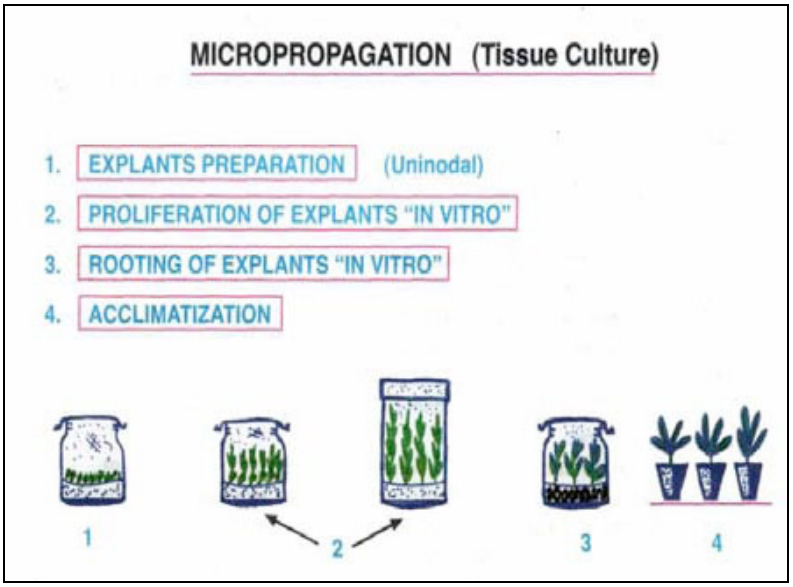

Fig. 4.1.1: Stages of "*in vitro*" Micropropagation. From Bartolucci *et* Dhakal, 1999

The factors that play an important role for the success of micropropagation process are:

1. the genotype of the plant donor;

2. the physiological conditions of donor material;
3. the source of explants;
4. the orientation and size of explants in culture;
5. the composition of the culture medium;
6. the balance between endogenous hormones and exogenous growth regulators;
7. the incubation conditions: quality and intensity of light, temperature, relative humidity and air quality;
8. the time interval of subculture.

4.1.3 - Medium composition and the primary role of hormones

The selection of the right culture medium is of fundamental importance in each phase of the *in vitro* propagation. Every medium includes mineral components, *macroelements* and *microelements*, and organic components such as carbohydrates, vitamins and growth regulators. Auxin, cytokinins and gibberellins are the hormones used in micropropagation technique and their concentrations and combinations depend on the plant species and culture phase. It is noteworthy to underlie that the ratio between different hormones rat her than their individual concentration is important during *in vitro* propagation and it differ in relation to the morphogenetic process which must be developed. Moreover, as a general rule, cells must be physiologically ready to receive hormone inductive signal and this physiological receptivity is called '*competence*' (Christianson *et* Warnick, 1988). However the time in which inductive signal is perceived can be extremely critical.

4.1.3.1 - Cytokinins and Shoot Development

Cytokinins are adenine derivatives, characterized by the ability to induce cell division in tissue culture, together with auxin. Natural cytokinins are: trans-zeatin (*4-hydroxy-3-methyltrans-2-butenylaminopurine*); IP (N^6-Δ^2*isopentenyladenine*) and dihydrozeatin (*4-hydroxy-3-methyl-trans-2-butenyl*). All adenine derivatives cytokinins replace the N^6 with an isoprenic chain derived from mevalonic acid pyrophosphate which undergoes decarboxilation, dehydration and isomerisation to give 2-isopentenyl pyrophosphate. Due to the presence of a double bond in the

isoprenic chain, the zeatin molecule has two configurations: *trans-isomer* and *cis-isomer*. The *trans-isomer* is the predominant configuration that occurs in nature (*Fig.4.1.2*). The *cis* isomer exhibits a low biological activity which can be explained by the existence of a *cis-trans isomerase* with high affinity to convert the *cis-zeatin* in the *trans-isomer* (*Fig.4.1.2*) (Mok *et al.*, 1992; Bassil *et al.,* 1993).

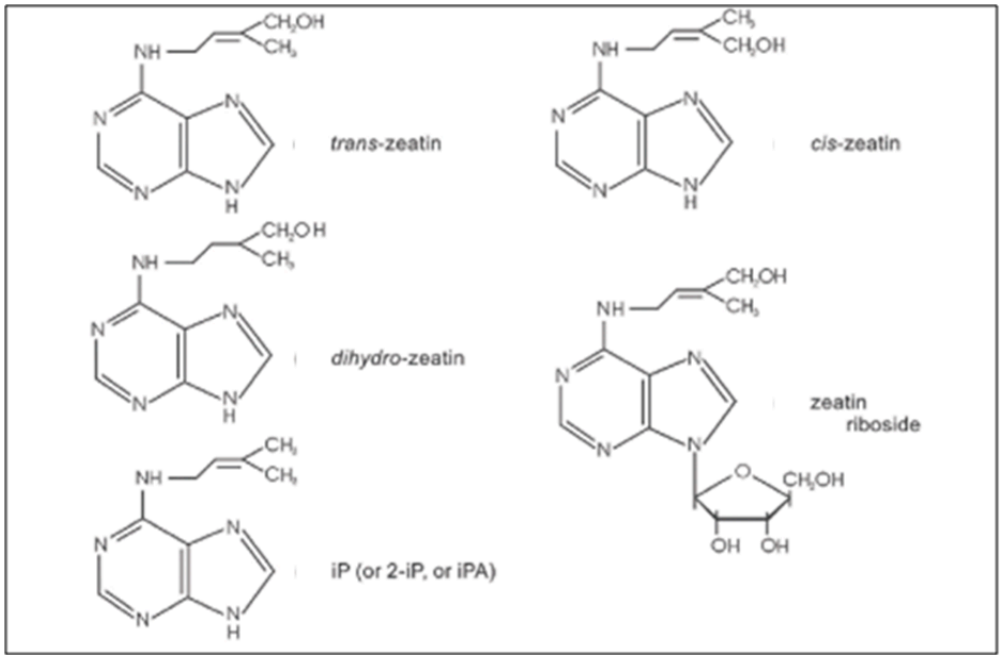

Figure 4.1.2: Molecular structure of cytokine. From George *et al.,* 2007

Cytokinins are known to play a major role in shoot development both *in vivo* and *in vitro* conditions (Davies, 1995). Concerning olive micropropagation, zeatin is the most utilized cytokinin for inducing shoot proliferation. It is required at high concentrations since olive plants grown *in vitro* are characterized by a strong apical dominance and such characteristic limits the potentiality for *in vitro* micropropagation (Rugini *et* Pannelli, 1993). Alternative synthetic cytokinins such as BAP (*6-Benzylaminopurine*) TDZ (*Thidiazuron*) and kinetin have be found to induce short shoots with a very large callus formation at its basis (Rugini, 1990; Grigoriadou *et al.,* 2002; Rugini *et* Baldoni, 2004). Moreover, synthetic cytokinins did not induce suitable proliferation rates and usually they induced explant hiperhidricity (Briccoli *et al.,* 2002; Garcìa-Fèrriz *et al.,* 2002). In some other *genera*, for example in *Galega vulgar* good results have been obtained by using a mixture of different cytokinins added of BAP and coconut water (Grigoriadou *et al.* 2002; Garcia-Fèrriz *et al.,* 2002;

Peixe *et al.,* 2007). With the aim to reduce the apical dominance of *Olea*, Mendoza-De Gyves and co-workers (2007) also used a combination of zeatin and Dikegulac (a compound that inhibit gibberellins biosynthesis in plants); this combination resulted apt to enhance the vegetative growth of *Canino, Frantoio* and *Moraiolo* olive cultivars. Thus, zeatin hormone remains the main cytokinin used for plant micropropagation *in vitro*.

4.1.3.2 - Auxin and Root Development

There is large evidence that auxin influence every aspect of plant growth and development (Davies, 1995).

IAA (*Indol-3-acetic acid*) is the most important natural Auxin, universally present in plants (Slovin *et al.,* 1999). IBA (*Indole-3-butyric acid*), identical to IAA except for two additional methylene groups in the side chain and originally classified as a synthetic auxin, is an endogenous plant compound with auxin-like activity (Epstein *et* Ludwig-Muller, 1993; Ludwig-Muller, 2000; Bartel *et al.,* 2001). In fact, like IAA, in *Arabidopsis,* exogenously supplied IBA inhibited root elongation and induced lateral and adventitious root formation (King *et* Stimart, 1998; Zolman *et al.,* 2000). In addition to the *indolic auxin*, a PAA (*Phenylacetic acid*) has been identified in plants with an auxin activity (Ludwig-Muller *et* Cohen, 2002).

Two main types of synthetic plant growth regulators with auxin-like activity have been described: NAA (*1-Naphthalacetic acid*) and 2,4-D (*2,4-Dichlorophenoxyacetic acid*)-related compounds. Both compounds exert auxin-like effects, such as the inhibition of root elongation and the promotion of lateral root development. The 2-NAA (*2-Naphthaleneacetic acid*) a NAA isomer, has little activity compared to the active 1-NAA (Thimann, 1977), while 2,4-DB (*2,4-Dichlorophenoxybutyric acid*) a 2,4-D derivative with two additional methylene groups in the side chain, elicits similar responses to those induced by 2,4-D treatment. There are evidences that IAA, 2,4-D, NAA and other synthetic compounds cause auxin-similar physiological responses, but distinct, although overlapping, changes in gene expression thus reflecting differences in their metabolism, transport, or interaction with auxin signaling (Pufky *et al.,* 2003).

In planta, auxin modulates different processes, such as trophic responses to light and gravity, general root and shoot architecture, organ patterning, vascular development, embryo development. In *in vitro* culture auxin is essential for somatic embryogenesis as well as for promoting organogenesis, and mainly adventitious root organogenesis (Davies, 1995).

4.1.3.3 - Adventitious Rooting

Adventitious roots are postembryonic roots, which arise from the stem, leaves and, in the root, from tissues others than pericycle (Geiss *et al.*, 2009). Adventitious roots can arise naturally from stem tissue under stressful environmental conditions; they may also be induced by mechanical damage. In these cases they arise from different cellular types, able to dedifferentiate and to acquire a meristematic fate. If the stem is old, the adventitious roots are formed by a parenchymatous ray cells, or by cells of the phloem in proximity to the cribro-vascular system (Casson *et.* Lindsey, 2003, and references therein).

In *in vitro* culture, adventitious roots can arise from callus (indirect organogenesis) or from specific cell types (direct organogenesis) (Li *et al.*, 2009). Their formation is a key step in the vegetative propagation, very essential for a successful production of elite clones which often involve economically important woody, horticultural and agricultural species. Therefore a lack of competence to form adventitious roots by cuttings is an obstacle for the vegetative propagation (De Klerk *et al.,* 1999; Céline *et al.,* 2006) .

Certainly, adventitious rooting capacity is a quantitative genetic trait but is also strongly influenced by culture conditions. Indeed, adventitious root formation is a complex process and includes three different phases, which exhibit a different hormone requirements (De Klerk *et al.,* 1999). In the first phase, there is the onset of cell proliferation that occurs via sequential and regulatory biochemical events culminating in cell cycle progression into S phase. This phase can also be identified as the phase of root induction. It follows the root initiation phase (second phase), in which the first anatomical modifications take place, and the protrusion phase (third phase) corresponding to the emergence of root primordial (Berthon, 1990; Heloir, 1996).

4.1.3.4 - Auxin and Adventitious Rooting

As already mentioned, auxin hormone is intimately involved in the adventitious rooting process (Wiesman *et al.*, 1988; Blakesley, 1994). In line with its major role, there is a tightly relationship between changes in endogenous auxin levels and the morpho-physiological phases of rooting (Heloir *et al.*, 1996). Usually, at the beginning of the rooting process high amounts of endogenous auxin are associated to a high rooting rate (Blažková *et al.*, 1997; Caboni *et al.*, 1997).

Auxins have been shown to be effective inducers of adventitious roots in many woody species (De Klerk *et al.*, 1999; Diaz-Sala *et al.*, 1996; Goldfarb *et al.*, 1998). Among the different auxins, IBA is more effective than IAA in inducing lateral and adventitious roots (Zolman *et al*., 2000). Biochemical analyses carried out in many plants and *Arabidopsis* genetic studies indicated that IBA acts primarily through its conversion into IAA (Bartel *et al*., 2001; Ludwig-Muller *et al.*, 2005). The best performance of IBA than IAA may be due to a higher stability, differences in metabolism, differences in transport or simply to its role as a IAA source. Recently, in *Arabidopsis thaliana* it has been demonstrated a differential role for IAA and IBA in the regulation of adventitious root formation from stem segments (Ludwig-Muller *et al.*, 2005). It has been suggested that IAA and IBA might interact in promoting adventitious root formation.

4. 2. MATERIALS AND METHODS

All the growth medium used in the different experiment were adjusted to 5.8 pH value autoclaved at 104 KPa and 121°C for 20 minutes, then enriched of growth regulators (sterilized by filtration) and a biocide mixture, the "Plant Preservative Mixture" (PPM™), at the final concentration of 0.1% (v / v).

The samples, placed on growth medium into appropriate sterile containers, were grown in a Phytotron at 24°C under a photoperiod of 16 h light /8 h darkness. The intensity of irradiation during the light period was 55 μmol m-2 s-1 PAR. The samples were transferred onto fresh medium at regular intervals. All culture procedures were done under a sterile laminar flow hood.

The mixtures in micro and macro elements and vitamins added to the media were:

a. the *Murashige and Skoog* mixture (Murashige *et* Skoog, 1962) referred to as MS basal medium.
b. the *Olive Rugini* mixture (Rugini, 1984) referred to as OR basal medium.

4. 2.1 - Seed Germination

Seeds (n= 700) were collected from *Oleaster* plants growing in open field at the locality "*Pietra del Demanio*", Civita (CS), Italy. Part (n= 580) of these seeds were subjected to vernalization at either 4°C or 10°C for 1, 3 and 4 weeks according to Voyiatzis (1995).

For germination test, unvernalized and vernalized seeds were suitably private of the woody endocarp and soaked for 2 days under running water. Subsequently, they were sterilized with 70% Ethanol (EtOH) for 1min and 5% NaOCl for 12min, supplemented with 0.1% Tween 20. Afterward seeds were washed thoroughly with sterile distilled water an treated with 1.5% PPM (v/v) supplemented with 50 mg/l of magnesium salts (chloride magnesium, sulphate magnesium, nitrate magnesium). The PPM treatment was performed for 7 hours under continuous stirring. Finally, the seeds were placed in culture on different mediums: A, T1 e T2 whose composition is

reported in Table 4.2.1. These media were selected on the basis of literature data on seed germination of *Olea* cultivars (Sakunasingh *et al.,* 2004).

Table 4.2.1.: Composition of culture medium used for seed germination of *Olea europaea* L.subsp. *europaea* var. *sylvestris*.

	A	T1	T2
***Culture Basal Medium* (gr/l)**	**Water**	**½ MS**	**MS**
NAA (µM)	--	5.37	0.27
Kinetin (µM)	--	--	13.95
GA3 (µM)	--	--	5.78
Sucrose (g/l)	--	20	30
Bacto-agar (%)	0.7	0.7	0.7
pH	5.8	5.8	5.8
PPM (%)	0.1	0.1	0.1

Pyrex tubes with 25mm of diameter and 150mm of height containing 20ml of medium were used and in each tube a single seed was placed.

Globally, 21 different conditions were tested (see *Table 4.3.1*). For each conditions two replicates were carried out and for each replicates 15 seeds were used. Germination process was monitored every 15 days for 2 months.

4.2.2 - Growth of Seedlings

Seedlings (n = 370), obtained in the two replicates of germination test, were at different times transferred into conical flasks of 250ml on 85ml of medium enriched or less of *trans*-zeatin (*Tab. 4.2.2*), in order to induce the vegetative growth. The applied zeatin concentration was 5mg/l (23µM), as reported in literature for vegetative growth of seedlings at this stage (Rugini, 1984; Brhadda *et al.,* 2003; Abousalim *et al.,* 2005). The vegetative growth was monitored for 45 days after which we proceeded with the subculture.

Table 4 .2.2: Composition of media culture used for "*in vitro*" growth of *Olea europaea* L.subsp. *europaea* var. *sylvestris* seedlings.

	MSZ_5	ORZ_5	MS	OR
***Culture Basal Medium* (gr/l)**	**4.4**	**4.02**	**4.4**	**4.02**
***Zeatin* (µM)**	23	23	--	--
Mannitol (gr/l)	30	30	30	30
Bacto-agar (%)	0.8	0.8	0.8	0.8
pH	5.8	5.8	5.8	5.8
PPM (%)	0.1	0.1	0.1	0.1

4.2.3 - Propagation of seedling-derived by micro-cuttings

Microcuttings with approximately the same length were excised from developed seedlings and transferred on two different medium, $MSZ_{2.5}$ and $ORZ_{2.5}$, whose composition is reported in *Table 4.2.3*. These microcuttings will be referred to as Smc_s (Seedling derived microcuttings). Both single node microcuttings without the apical bud (Smc_1) (n=100) and microcuttings with two nodes and presenting the apical bud (Smc_2) (n=120) were used. Three independent replicates were performed and subcultures were made after 45 days.

Table 4.2.3: Composition of media culture used for "*in vitro*" propagation of Smc_s of *Olea europaea* L.subsp. *europaea* var. *sylvestris*.

	$MSZ_{2.5}$	$ORZ_{2.5}$
***Culture Basal Medium* (gr/l)**	**4.4**	**4.02**
***Zeatin* (µM)**	11.5	11.5
Mannitol (gr/l)	30	30
Bacto-agar (%)	0.8	0.8
pH	5.8	5.8
PPM (%)	0.1	0.1

4.2.4 - *In vitro* rooting of Smc_s microcuttings

For rooting tests we used actively growing Smc_s, obtained after two subcultures (30 days for each subculture) on ORZ_5 medium (*Tab. 4.2.2*), according to the procedure above described.

Smc_s with 3.0 nodes and bearing the apical bud were excised and transferred, under different conditions, into different media as reported in Table 4.2.4a:

Table 4.2.4a: Rooting conditions tested on Smc_s of *Olea europaea* L.subsp. *europaea* var. *sylvestris*

Rooting condition	*Pre-treatment*	Medium
T_1	---	ORm
T_2	O/N → 3 mg/l (14.7 µM) IBA	ORm
T_3	20'' → 3 mg/l (14.7 µM) IBA	ORm + 0.5 mg/l (2.4µM) IBA
T_4	20'' → 3 mg/l (14.7 µM) IBA	ORm + 1 mg/l (4.9µM) IBA

The "*Pre-treatment*" consisted into the immersion of basal portion of Smc_s in a sterile solution of 3 mg/l *Indol-3-butyric acid* (IBA) for 20 seconds or overnight (O/N). The medium used was an OR modified medium (ORm) enriched or not with IBA at different concentrations.

The composition of ORm medium is reported in Table 4.2.4b.

Table 4.2.4b: a) Composition of ORm medium used for the rooting tests conducted on seedling microcuttings of *Olea europaea* L.subsp. *europaea* var. *sylvestris*

OR medium modified (ORm) a) basal composition	
OR *basal medium* (gr/l)	4.02
Sucrose (gr/l)	30
Phytagel (mg/l)	2.5
pH	5.8
PPM (%)	0.1

b) darkening Solution	
Activated Charcoal (gr/l)	4
Bacto-agar (%)	0.8
pH	5.8
PPM (%)	0.1

In particular ORm composition included also a Darkening solution which was added to solidified basal composition in order to obscure the medium surface.

The cultures were weekly monitored for 4 week. Two independent replicates were carried out by using n = 25 samples for each test and for each replicate.

4.2.5 - Histological analysis of adventitious roots

Histological analysis was performed on n = 5 adventitious roots, obtained from Smc_s subjected to rooting tests previously described (*Tab. 4.2.4a*). The anatomy of *Oleaster* adventitious root was compared to that of embryonic one. For this purpose, n= 5 embryonic roots were excised from 28 days old seedlings obtained from seeds and germinated as above described.

Preparation of glass slides

Roots were excised and rapidly fixed in a solution of glutaraldehyde 0.5% (v/v) and paraformaldehyde 3% (w/v) in PBS buffer (135 mM NaCl, 2.7mm KCl, 1.5mM KH_2PO_4, 8mM K_2HPO_4, pH 7.2). This step was performed for 3hours at 4 °C. To optimize the fixation, was carried out the degassing of samples with a vacuum pump.

Subsequently samples were washed with distilled water for 30' and dehydrated in an increasing series of alcohols (EtOH 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60% , 70%, 80%, 95%, 100%) at intervals of 30' each one.

Afterwards, samples were transferred into a infiltration solution consisting of EtOH and resin Tecnovitt 8100® (BIO-OPTICA), in increasing ratios (3:1, 1:1, 1:3) until resin 100%. Finally samples were polymerized and sectioned to a 4µm of

thickness with a microtome Leica RM 2155.The sections were placed on glass slides and left to dry on a thermostated plate at the temperature of 37°C for 12h .

The longitudinal sections of adventitious and embryonic roots were stained with "Feulgen" reaction.

The "Feulgen" reaction provides a bland hydrolysis of samples with hydrochloric acid (1N) at a temperature of 60°C for 7 minutes and the staining with Schiff's reagent. The hydrolysis determine the break of hydrogen bonds located between the purine and pyrimidine bases of DNA and expose the aldehyde functions of the sugar associated (deoxyribose which is an aldohexose), to the Schiff's reagent. The Schiff's reagent or acid-sulphurous fucsino is so named because the fuchsin, a red-purple dye is decolorized with sulphurous acid. The red-purple color reappears by the reaction of Schiff's reagent with the aldehyde functions. The staining reaction was conducted for 1 hour in the dark.

At the end of the treatment with Schiff's reagent, the glass slides were washed to remove excess of dye, were dried on a thermostated plate at the temperature of 37°C for 3 hours and counter-stained with a solution of Azur II 0.5% (w/v).

Finally, the glass slides were washed, dried on a thermostated plate at the temperature of 37 °C for 12h and closed permanently with Canadà balsam and coverslip. The glass slides as processed were observed with the Leica DMRB microscope and the images were acquired with the program "Leica Application Suite V 3.2.0."

4.2.6 - *In vitro* propagation of stem cuttings derived by field-grown plants

Cuttings were excised from *Oleaster* plants growing in open fields, along Tirrenian coast at Cetraro (CS), Italy. In order to obtain a single clone, cuttings were randomly collected from a single plant. Single node cuttings were excised from branch region spanning from the third to the seventh node. These microcuttings will be referred to as Stc_s (Stem cuttings).

Sterilization

Preliminarily, it was setted up an efficient sterilization protocol which included two phases:

Phase 1 – After excision of leaves, cuttings were accurately washed under running water for 2 hours. Afterwards they were washed for 10 min in a 0.3% water solution of Ausilab-101, a commercial detergent, and then under running water for 10 min in order to remove residual detergent. Finally, they were immersed for 30 minutes in ascorbic acid (150 mg / l) and citric acid (100 mg / l) water solution in order to limit oxidative processes (Brhadda *et al.*, 2003). The residual acids were removed through several washings in sterile distilled water.

Phase 2 - Cuttings were subjected to chemical sterilization in 70% EtOH for 1min, afterwards they were treated with 2% NaOCl supplemented with 0.1% tween 20 for 15 min and washed thoroughly with sterile distilled water. Finally, cuttings were subjected to 1.5% (v/v) PPM supplemented with 50 mg/l of magnesium salts as above described. The PPM treatment was performed for 1 hour, under continuous stirring.

Induction of axillary buds

To induce the vegetative growth of axillary buds, sterilized Stc_s were transferred on three different media: OR (without hormone) whose composition is that effectively used for *in vitro* growth of Smc_s (*Tab. 4.2.2*), $ORZ_{0.5}$ and $ORZ_{2.5}$ enriched of zeatin with 0.5 and 2.5 mg/l , respectively (*Tab. 4.2.5*).

Tab. 4.2.5: Growth medium tested for "*in vitro*" stabilization of **Stc_s** of *Olea europaea* L.subsp. *europaea* var. *sylvestris*

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Media tested for vegetative growth of Stc axillary buds		
Media tested	**Composition**	**Hormonal contribution**
OR	OR	--------
$ORZ_{0.5}$	OR	0.5 mg/l (2.3 µM) of *zeatin*
$ORZ_{2.5}$	OR	2.5 mg/l (11.4 µM) of *zeatin*

Stc_s were placed into pyrex tubes of 25mm diameter and 150mm height containing 20ml of medium. In each tube was placed a single node cutting and cultures were weekly monitored for 45 days. Two independent replicates were carried out; n= 50 Stc_s were used for each test and for each replicate.

Elongation of sub-cultured axillary shoot

Axillary shoot cuttings (Asc)**,** about 8 mm in length and exhibiting 3 nodes, obtained from Stc_s grown on $ORZ_{0.5}$ medium, as above described, were excised and sub-cultured on OR medium (See *Tab. 4.2.2* for composition) supplemented or not with zeatin at different concentrations (*Tab.4.2.6*).

Tab. 4.2.6: Different media tested for Elongation of Asc of *Olea europaea* L.subsp. *europaea* var. *sylvestris*..

Elongation tests	Media composition
OR	OR
ORZ_2	OR + 2 mg/l (9,12 µM) zeatin
$ORZ_{2.5}$	OR + 2,5 mg/l (11,4 µM) zeatin
ORZ_3	OR + 3 mg/l (13,8 µM) zeatin
ORZ_5	OR + 5 mg/l (23 µM) zeatin

The cultures were monitored each two weeks up to 45 days. Two replicates were performed; n = 15 axillary shoots were used for each test and for each replicate

4. 3. RESULTS

4. 3.1 - Seed Germination and seedling growth

Effect of Vernalization on seed germination

Before germination test, *Oleaster* seeds were subjected to vernalization at different temperatures, for different periods, and then germinated on different medium as illustrated in M&M section.

As reported in Table 4.3.1. un-vernalized seeds placed on agar (A), germination process started after approximately 60 days from the beginning of culture and the maximum germination value was 46% ± 3 (*Tab. 4.3.1*).

Under the same conditions, prolonged vernalization treatments ($V1_{3,4}$ and $V2_{3,4}$) induced a significant advance in the germination time, as well an increase in the percentage of germinated seeds. In fact, whatever temperature was applied, seeds which were vernalized for 3-4 weeks ($V1_{3,4}$ and $V2_{3,4}$) started to germinate after 15 days from placing in culture, reaching a 68% ±3 value of germination percentage (*Tab. 4.3.1*; *Fig. 4.3.1a*). No differences were instead observed between seeds germinated on agar and seeds germinated on either nutrient-enriched (T1) or nutrient-plus-hormone-enriched (T2) medium (*Tab. 4.3.1*; compare A vs T; A+V vs T+V).

About one month after germination, embryos exhibited expanded and intensely green-colored cotyledons, ready to undergo autotrophic life (*Fig. 4.3.1a*). At this stage embryos were isolated from the endosperm and transferred on fresh growth medium.

Table 4.3.1.: Effects of vernalization pre-treatments (V) and medium composition (A, T1, T2) on seed germination of *Olea europaea* L. subsp. *europaea* var. *sylvestris*. A= Agar; T1,T2= differentially enriched MS medium;$V1_1$-$V1_3$-$V1_4$: Vernalization at 4 ° C for 1-3-4 weeks respectively; $V2_1$-$V2_3$-$V2_4$: Vernalization at 10 ° C for 1-3-4 weeks respectively. The results represent the average of two different experiments. The analysis of obtained data was performed using the program Stat Work.

Test	Germination (%)	Germination start up (days)
A	46±2	60±1
A+V1$_1$	45±2	61±2
A+V1$_3$	68±3	15±2
A+V1$_4$	69±2	15±1
A+V2$_1$	46±4	60±2
A+V2$_3$	68±2	15±1
A+V2$_4$	67±2	16±1
T1	45±3	64±2
T1+V1$_1$	48±2	58±1
T1+V1$_3$	68±2	15±2
T1+V1$_4$	66±3	16±1
T1+V2$_1$	48±1	59±1
T1+V2$_3$	69±2	15±2
T1+V2$_4$	70±3	18±3
T2	46±3	61±2
T2+V1$_1$	48±2	60±1
T2+V1$_3$	70±3	20±3
T2+V1$_4$	68±3	16±2
T2+V2$_1$	47±2	63±1
T2+V2$_3$	68±3	18±3
T2+V2$_4$	66±2	15±2

Therefore, the obtained results suggest that the vernalization treatments rather than the enrichment of medium, promoted seed germination (*Tab. 4.3.1*). It is likely that reserves and hormones accumulated in *Oleaster* seeds are sufficient to support germination process. Based on these results, in all the subsequent experiments seed germination was carried out at **A+V1$_3$** condition.

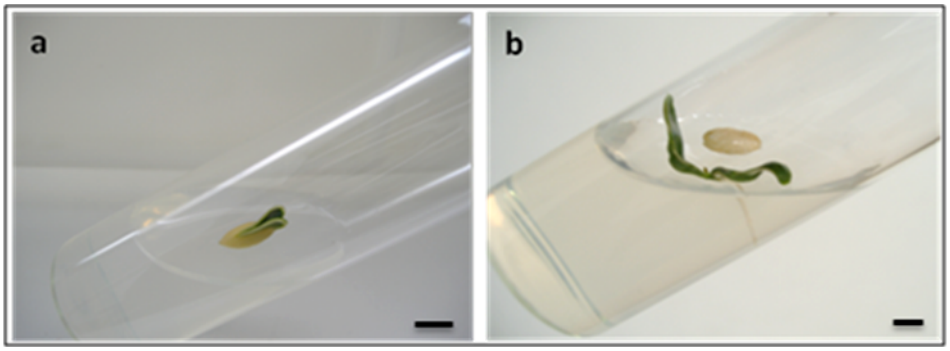

Fig. 4.3.1a: Seeds of *Olea europaea* L. subsp. *europaea* var. *sylvestris* subjected to vernalization treatments at 4°C for 3 weeks and placed to germinate on **A** medium **a)** 15 days after the start-up culture; **b)** 30 days after the start-up culture. Bar: a = 0.53 cm; b = 0.4 cm.

Seedling growth

The seedlings obtained from germination test were, at different times, transferred on different medium: MS and OR, without hormones, and MSZ$_5$ and ORZ$_5$ enriched with 5 mg/l zeatin. Along a 45day period, the seedlings growing on MS and OR mediums did not show any vegetative growth, while on zeatin-added medium seedlings underwent to a rapid vegetative growth, quite comparable on MSZ$_5$ vs ORZ$_5$. In fact, in both cases, after 7-10 days from transferring on the new medium, 84% of seedlings exhibited new formed leaves and after 5 weeks the plantlets reached a stage with 3-4 nodes. However, under these growth conditions, callus formation was observed at the base of the stem, clearly evidencing an unsuitable hormonal balance (*Fig. 4.3.1b*).

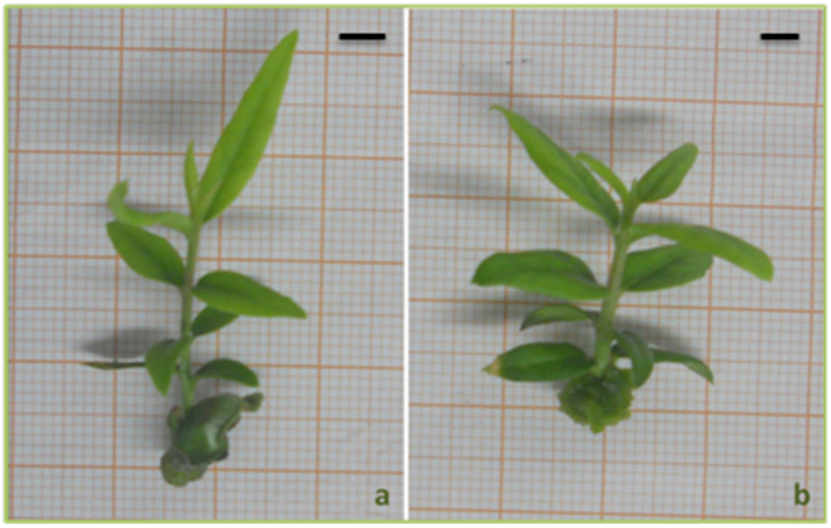

Fig 4.3.1b: Seedlings of *Olea europaea* L. subsp. *europaea var. sylvestris* after 6 weeks on (**a)** ORZ_5 and (**b)** MSZ_5 mediums for vegetative growth. Bar: a = 0.55 cm; b = 0.5 cm.

4.3.2 - *In vitro* propagation of microcuttings derived by seedlings

Microcuttings with approximately the same length (4-5 mm) were excised from developed seedlings: from here, we will refer to as seedling-derived microcuttings (Smc_s). In particular both uninodal segments lacking of apical bud (Smc_1) and apical binodal segments (Smc_2) were excised and transferred on medium at different zeatin concentrations: $MSZ_{2.5}$ and $ORZ_{2.5}$ (*Table 4.2.2b*). Note that in the case of Smc_2, the presence of the apical bud inhibited axillary bud development and consequently secondary shoot formation. For this reason vegetative growth of Smc_1 and Smc_2 was monitored by using different parameters (*Table 4.3.2*). In particular, for Smc_1 we evaluated *budding percentage* (= number of cuttings with open buds / total number of cuttings x100), *shooting percentage* (= number of cuttings with lateral shoots / total number of cuttings x100), shoot *length*, while for Smc_2 we evaluated: *cutting length, number of nodes*, *internode length*; *basal leaf length* (*Tab. 4.3.2b*).

As reported in tables 4.3.2 Smc_1 and Smc_2 showed an appreciable vegetative activity, without any symptom of vitrification, whatever medium (Ms vs OR) was used. Indeed, Smc_2 elongated forming new nodes (*Tab. 4.3.2b*), as well as appreciable percentages in both budding and shooting processes were exhibited by

Smc_1. Moreover, light but significant differences were observed for most of the analyzed parameters when comparing $MSZ_{2.5}$ vs $ORZ_{2.5}$ medium: this latter allowed a somehow higher vegetative growth.

Table 4.3.2: Effect of different medium ($MSZ_{2.5}$ and $ORZ_{2.5}$) on the vegetative growth of seedling-derived microcuttings (Smc) of *Olea europaea* L. subsp. *europaea* var. *sylvestris* after 45 days of *in vitro* culture. (a) Uninodal segments lacking of apical bud (Smc_1); (b) apical binodal segments (Smc_2). Composition of $MSZ_{2.5}$ and $ORZ_{2.5}$ is reported in Table 4.2.2b. Results represent the average value of three independent replicates. For statistical evaluation of data, Analysis of Variance (ANOVA) followed by Turkey test was applied. The percentage values were analyzed using Fisher test. Values marked with different letters show statistically significant differences for $P \leq 0.05$.

(a)

Medium	Sample Number	Budding (%) n° of cuttings with open buds / n° total of cuttings x100		Shooting (%) n° of cuttings with lateral shoots / n° total of cuttings x100		Shoot length (mm)		Node Number	
$MSZ_{2.5}$	50	66.7	a	37.5	a	9.17	a	1.91	a
$ORZ_{2.5}$	50	69	a	48.05	b	10.49	a	3.08	b

(b)

Medium	Sample Number	Cutting length (mm)		Node Number		1st internode length (mm)		2^{nd}-3^{rd}internode lenght (mm)		Basal leaf length (mm)	
$MSZ_{2.5}$	60	10.60	a	3.5	a	3.44	a	3.64	a	12.2	a
$ORZ_{2.5}$	60	15.15	b	3.5	a	4.486	a	5.039	b	15.1	b

The *in vitro* actively growing microcuttings obtained through this phase were used for several subculture cycles.

4.3.3 - *In vitro* rooting of actively growing Smc_s

The first tempt to induce adventitious root formation was performed by maintaining actively growing Smc_s on the same medium for a prolonged period. After 3-4 months from the beginning of *in vitro* culture, only 10% of Smc_s growing on $ORZ_{2.5}$ medium were able to produce adventitious roots, while vegetative growth

proceeded in both $ORZ_{2.5}$ and $MSZ_{2.5}$ medium at the optimal rate previously observed. It is interesting to note that the rooted plantlets derived from **Smc_s** were successfully acclimated in pots at 23 ° C (*Fig. 4.3.2*). Some of them were used for molecular analysis (see Section C par. 6.2.1) Currently, the remaining plants grow in open field at the Botanical Garden of University of Calabria (Arcavacata di Rende, CS).

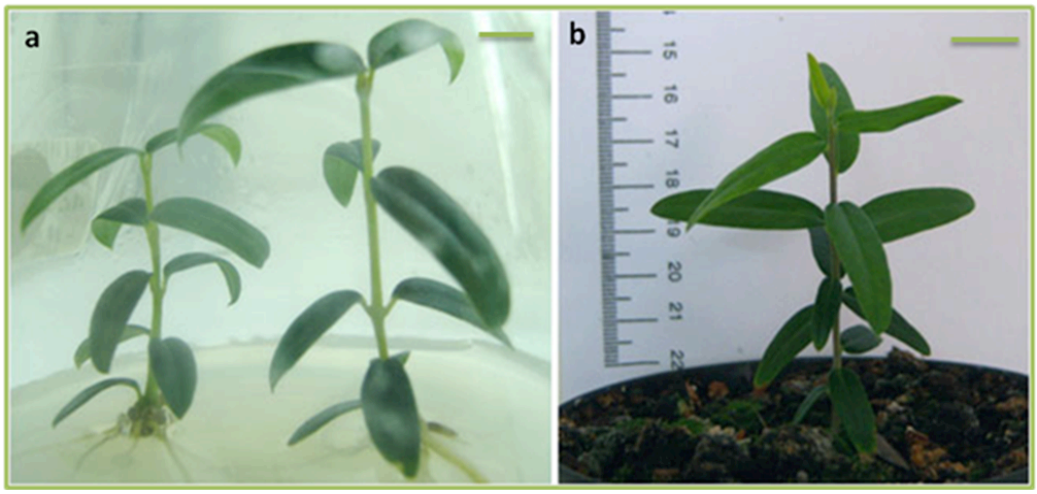

***Fig 4.3.2*: a)** Rooted Smc_1 of *Olea europaea* L. subsp. *europaea* var. *sylvestris* after 3 months of *in vitro* culture on $ORZ_{2.5}$ medium**; b)** *in vitro* rooted plantlets after 1 months from acclimatization on pots. Bar: a = 0.97 cm; b = 1.1 cm.

To improve rooting capacity of Smc_s and above all to shorten the period for inducing adventitious root formation, different protocols were applied. In particular, we used OR basal medium modified as reported in M&M Section (see *Tab. 4.2.4b*), adding in some cases either a short (20s) or long-lasting (overnight) pre-treatment with 3 mg/l IBA. To briefly summarize the different treatments, the following conditions were used: T_1 (no pretreatment + medium deprived of hormones); T_2 (long-lasting pretreatment + medium deprived of hormones); T_3 (short-pre-treatment + medium enriched with 0.5 mg/l IBA); T_4 (short-pre-treatment + medium enriched with 1.0 mg/l IBA).

For these rooting tests we used actively growing Smc_s, with 3.0 nodes and bearing the apical bud, obtained after two subcultures on ORZ_5 medium, as described in M&M. Smc_s cultures were weekly monitored and the following parameters were

evaluated: rooting percentage (number of rooted cuttings / total cuttings in culture x 100), number of roots for cutting and root length (*Tab 4.3.3*).

Table 4.3.3: (a) Rooting percentage (number of rooted cuttings / total cuttings in culture x 100) , (b) number of roots for cutting and (c) root length of seedling-derived microcuttings (Scm_s) of *Olea europeae* L. subsp. *europaea* var. *sylvestris* under different conditions of *in vitro* culture. T_1 (no pretreatment + medium deprived of hormones); T_2 (long-lasting pretreatment with 3 mg/l IBA + medium deprived of hormones); T_3 (short-pre-treatment with 3 mg/l IBA + medium enriched with 0.5 mg/l IBA); T_4 (short-pre-treatment with 3 mg/l IBA + medium enriched with 1.0 mg/l IBA). The results represent the average value of two independent replicates. For statistical evaluation of data, Analysis of Variance (ANOVA) followed by Turkey test was applied. The percentage values were analyzed using Fisher test. Values marked with different letters show statistically significant differences for $P \leq 0.05$.

(a)

Rooting conditions	**Rooting percentage (%)**							
	7gg		***14gg***		***21gg***		***28gg***	
T_1	0	a	44.4	a	66.7	a	66.7	a
T_2	0	a	25	b	25	b	25	b
T_3	0.2	a	55.5	a	69.8	a	69.8	a
T_4	0.3	a	80	c	80	c	90	c

(b)

Rooting conditions	**N° of roots for cutting**							
	7gg		***14gg***		***21gg***		***28gg***	
T_1	0	a	1	a	1.16	a	2.3	a
T_2	0	a	3.5	a	4	b	4.5	b
T_3	1	a	2	a	2.1	a	2.3	a
T_4	1.3	a	2.3	a	2.6	a	2.7	a

(c)

Rooting conditions	Root length (mm)							
	7gg		*14gg*		*21gg*		*28gg*	
T_1	0	a	13.5	a	28.86	a	37.29	a
T_2	0	a	12	a	19.88	a	20.22	b
T_3	2.00	ab	12.6	a	24.00	a	35.14	ab
T_4	3.38	b	21.16	b	43.24	b	56.33	c

The results reported after 28 days of *in vitro* culture are summarized in figures *4.3.3.1* and *4.3.3.2*. As for as rooting percentage is concerned, the best result (90%) was obtained at T_4 condition, consisting in a short-pre-treatment and in the use of growth medium with the highest IBA concentration. Relevant and quite similar values (66.7 and 69,8%, respectively) were also reported both in total absence of IBA (T_1) and at T_3 condition, consisting in a short-pre-treatment and the use of growth medium with the lowest IBA concentration. By contrast, the long-lasting pre-treatment with IBA followed by a growth medium deprived of hormone (T_2) resulted into only 25% rooting percentage (*Tab 4.3.3a; Fig 4.3.3.1*).

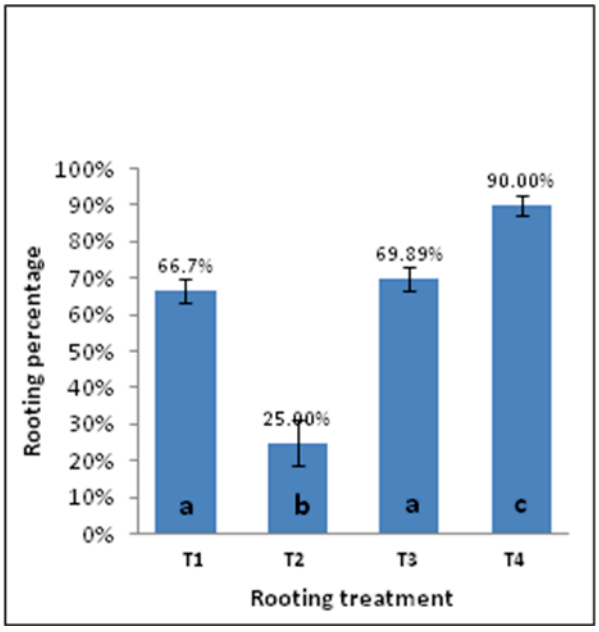

Figure 4.3.3.1: Rooting percentage (number of rooted cuttings / total cuttings in culture x 100) of *Olea europeae* L. subsp. *europaea* var. *sylvestris* Smc_s, under different conditions of *in vitro* culture:

T_1 (no pretreatment + medium deprived of hormones); T_2 (long-lasting pretreatment with 3 mg/l IBA + medium deprived of hormones); T_3 (short-pre-treatment with 3 mg/l IBA + medium enriched with 0.5 mg/l IBA); T_4 (short-pre-treatment with 3 mg/l IBA + medium enriched with 1.0 mg/l IBA)**.** The results represent the average value of two indipendent replicate. For statistical evaluation of data, Analysis of Variance (ANOVA) followed by Turkey test was applied. The percentage values were analyzed using Fisher test. Values marked with different letters show statistically significant differences for $P \leq 0.05$.

Concerning the number of roots for cutting induced at the different culture conditions, an opposite effect was observed compared to the rooting percentage. Namely, in this case the highest root number was promoted by T_2 condition, whereas comparable lower values were observed under the other conditions (*Figs 4.3.3.2 and 4.3.3.3).*

However, taking into account the root length, once again the greatest and the lowest effectiveness was associated to T_4 and T_2 conditions, respectively. Namely, adventitious roots developed from Smc_s after 28 days of culture at T_4 condition reached a length of 56.33 ± 1.98 mm while at T_2 condition root length was 20.22 ± 1.33 mm (*Tab 4.3.3c; Figs 4.3.3.2 and 4.3.3.3*).

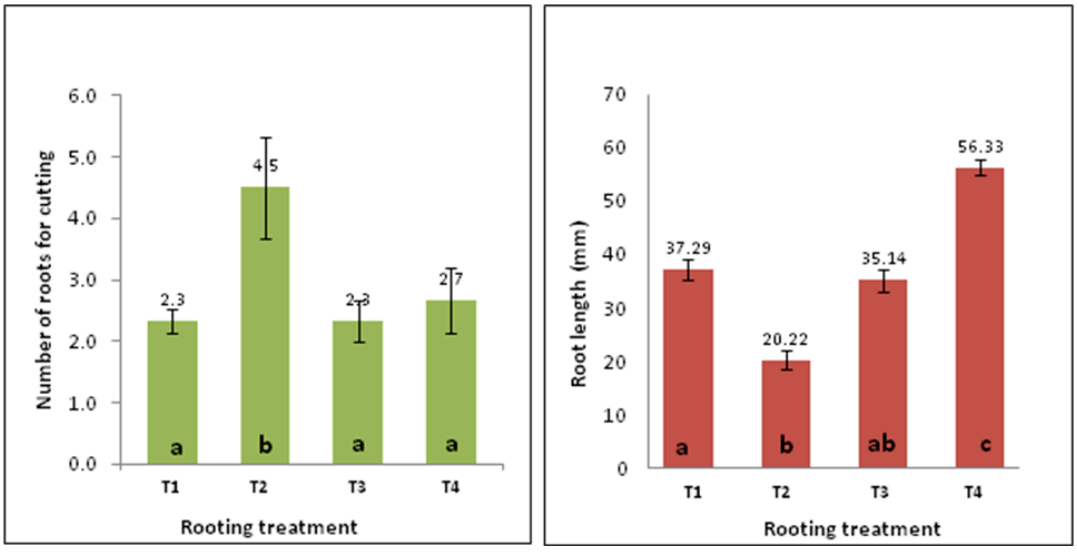

Figure 4.3.3.2: Number of roots for cutting and root length of *Olea europeae* L. subsp. *europaea* var. *sylvestris* Smc_s, under different conditions of *in vitro* culture: T_1 (no pretreatment + medium deprived of hormones); T_2 (long-lasting pretreatment with 3 mg/l IBA + medium deprived of

hormones); T_3 (short-pre-treatment with 3 mg/l IBA + medium enriched with 0.5 mg/l IBA); T_4 (short-pre-treatment with 3 mg/l IBA + medium enriched with 1.0 mg/l IBA). The results represent the average value of two independent replicates. For statistical evaluation of data, Analysis of Variance (ANOVA) followed by Turkey test was applied Values marked with different letters show statistically significant differences for $P \leq 0.05$.

No differences were observed between roots formed by Smc_s grown under T_1 and T_3 conditions (*Tab 4.3.3*).

The described differences in root number and length are clearly illustrated in Figure 4.3.3.3.

Figure 4.3.3.3: Features of *Olea europaea* L. subsp. *europaea* var. *sylvestris* Smc_s, under different conditions of in vitro culture. **(a)** T_1 (no pretreatment + medium deprived of hormones); **(b)** T_2 (long-lasting pretreatment with 3 mg/l IBA + medium deprived of hormones); **(c)** T_3 (short-pre-treatment with 3 mg/l IBA + medium enriched with 0.5 mg/l IBA); **(d)** T_4 (short-pre-treatment with 3 mg/l IBA + medium enriched with 1.0 mg/l IBA). Bar: a = 0.8 cm; b = 0.6 cm; c = 0.85 cm; d = 0.96 cm.

4.3.4 - Histological analysis of adventitious roots developed during "*in vitro*" Smc_s propagation.

To obtain some insights into their pattern formation, adventitious roots, developed during rooting phase, were analyzed at histological level and compared with embryonic roots (*Fig. 4.3.4*). It was evident that, at the level of root apex, tissue organization was quite comparable in adventitious and embryonic roots, in that it was possible to distinguish a meristematic zone covered by calyptra, followed in basipetal direction by elongation zone (*Fig. 4.3.4* a, b). However, it was possible also to notice

some interesting differences dealing with the presence, in adventitious roots, of a calyptra smaller in size (*Fig. 4.3.4* b, f) and numerous root hairs present in the elongation zone compared to embryonic root (*Fig. 4.3.4* b, d). This latter feature clearly highlighted an early differentiation of protoderm cell line in adventitious vs embryonic roots.

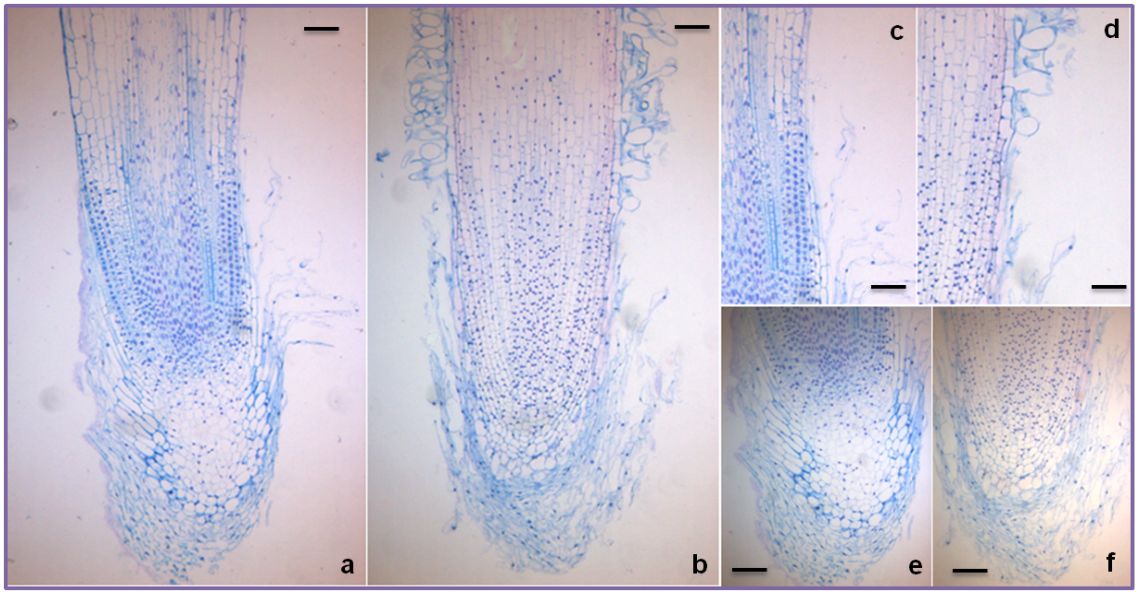

Figure 4.3.4: Longitudinal sections of embryonic (a, c, e) and adventitious roots (b, d, f) of *Olea europaea* L. subsp. *europaea* var. *sylvestris*, colored by Feulgen and counter-stained with Azur II. The adventitious roots were obtained from in vitro rooted microcutting (Smc_s). The embryonic roots come from *in vitro* germinated seeds. Bar.: a = 61 mm; b = 75mm; c – d = 40 mm; e – f = 27 mm

4.3.5 - Vegetative growth and acclimatation in pots of rooted Smc_s

It is worthy noting that differences in root growth were not associated to differences in vegetative growth, as evidenced by the comparable length reached by Smc_s at all the analyzed conditions (*Tab 4.3.4*).

Table 4.3.4: Length of *Olea europaea* L. subsp. *europaea* var. *sylvestris* Smc_s, growing *in vitro* under different rooting conditions of culture**:** T_1 (no pretreatment + medium deprived of hormones); T_2 (long-lasting pretreatment with 3 mg/l IBA + medium deprived of hormones); T_3 (short-pre-treatment with 3 mg/l IBA + medium enriched with 0.5 mg/l IBA); T_4 (short-pre-treatment with 3 mg/l IBA + medium enriched with 1.0 mg/l IBA). The results represent the average value of two

indipendent replicate. For statistical evaluation of data, Analysis of Variance (ANOVA) followed by Turkey test was applied Values marked with different letters show statistically significant differences for $P \leq 0.05$.

Rooting conditions	Cuttings length (mm)							
	7gg		*14gg*		*21gg*		*28gg*	
T_1	16.89	a	17	a	18.11	a	24.48	a
T_2	18.25	a	18.5	a	18.5	a	21.5	a
T_3	18.44	a	18.44	a	20.33	a	22.11	a
T_4	19.1	a	19.4	a	22.1	a	27.3	a

Finally, rooted Smc_s were transferred and successfully acclimated in pots at a temperature of 23 ° C (*Fig. 4.3.5*).

***Figure 4.3.5*:** Rooted microcuttings of *Olea europaea* L. subsp. *europaea* var. *sylvestris* after 50 days of acclimatization in pots Bar: a = 1.1 cm; b = 1.03 cm.

4.3.6 - *In vitro* propagation of stem cuttings derived by field-grown plants

Single node stem cuttings (Stc_s) excised from plants grown in open fields were used.

Sterilization of Stc_s

The creation of a sterile culture is the most difficult step in the micropropagation of woody plants. Downstream of an accurate preliminary screening, an efficient two-step procedure was set up, whose details have been reported in the M&M Section. This method has been effective both in controlling bacterial and fungal contamination without affecting Stc_s growth and preventing their oxidation. In fact, through this method it was achieved a 96% of un-contaminated Stc_s culture.

Vegetative Growth of axillary buds from Stc_s

For inducing vegetative growth of axillary buds from **Stc_s**, three different growth medium were tested: OR, $ORZ_{0.5}$, and $ORZ_{2.5}$ whose composition is described the *Tables 4.2.2 and 4.2.5*. of M&M Section.

Stc_s, culture were weekly monitored and at 45 day of culture the following parameters were evaluated: *budding percentage* (number of cuttings with open buds / total number of cuttings x100), *shooting percentage* (number of cuttings with axillary shoots / total number of cuttings x100), shoot *length* (*Tab 4.3.5*).

Table 4.3.5: Vegetative growth of stem cutting (Stc_s) of *Olea europaea* L. subsp. *europaea* var. *sylvestris*, excised from grown-field plants and *in vitro* cultured for 45 days. OR (medium without hormone); $ORZ_{0.5}$ (medium enriched with 0.5 mg/l of zeatin); $ORZ_{2.5}$ (medium enriched with 2.5 mg/l of zeatin). The results represent the average value of two independent replicates. For statistical evaluation of data, Analysis of Variance (ANOVA) followed by Turkey test was applied. The percentage values were analyzed using Fisher test. Values marked with different letters show statistically significant differences for $P \leq 0.05$.

Growth medium	**% Budding** n° of cuttings with open buds / total number of cuttings x100		**% Shooting** n° of cuttings with axillary shoots / total number of cuttings x100		**Shoot Length (mm)**	
OR	6	a	0	a	0	a
$ORZ_{0.5}$	85	b	47.5	b	4.26	b
$ORZ_{2.5}$	69	c	5.4	c	3.33	b

The obtained results highlighted that in the absence of hormones (OR), Stc_s, underwent to a very low vegetative growth. In fact, after 45 days from culture start-up, budding was observed only in 6% of cultured Stc_s. Moreover, not any of open buds was able to produce shoots (*Tab 4.3.5*). Apparently, this result is quite different from that obtained by propagating Smc_s on the same medium (*Tab 4.3.2a*) but it must be underlined that Smc_s derived from seedlings grown for 45 days on zeatin-enriched OR medium.

Accordingly, the addition of zeatin to the growth medium positively and significantly influenced the vegetative growth: in fact, the percentage of cuttings with open buds proved to be equal to 85% and 69% on $ORZ_{0.5}$, and $ORZ_{2.5}$ medium, respectively (*Table 4.3.5*). However, $ORZ_{0.5}$ medium resulted to be the most effective by significantly promoting also the development of axillary shoots: in fact 47.5% of the cuttings developed axillary shoots, while the percentage of shooting on the medium $ORZ_{2.5}$ was significantly lower (5.4%) compared to $ORZ_{0.5}$ (*see Table 4.3.5*).

The length of the formed shoots was also monitored and it was found to be greater on $ORZ_{0.5}$, vs $ORZ_{2.5}$, but in this case the differences between the two culture medium were less striking (*Tab. 4.3.5*). Photographic evidence of the described differences are provided in figure 4.3.6.

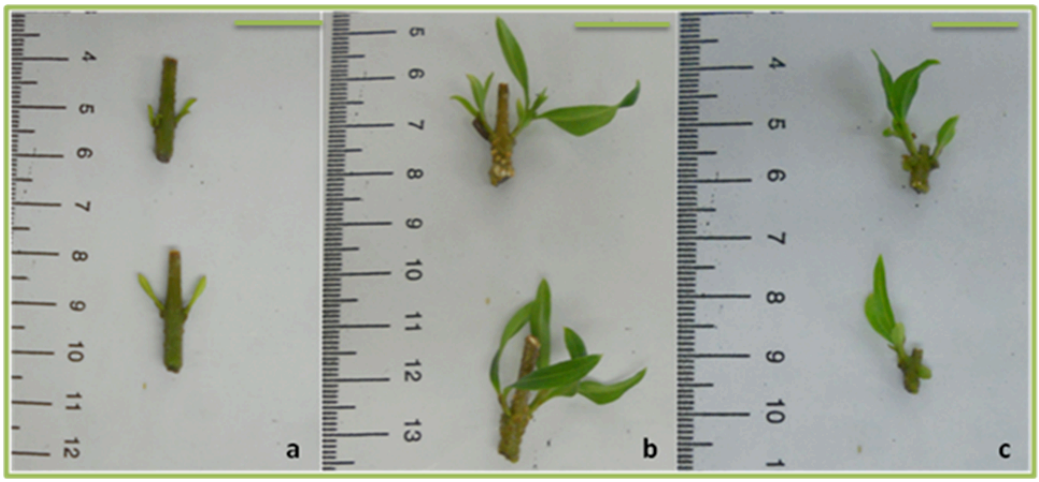

Fig 4.3.6: Vegetative growth of stem cuttings (Stc_s) of *Olea. Europeae* L. subsp. *europaea* var. *sylvestris* excised from field-grown plants and *in vitro* cultured on three different mediums for 45 days. a) OR *medium* without hormones; b) $ORZ_{0.5}$ *medium* enriched with 0.5 mg/l of

zeatin; c) $ORZ_{2.5}$ *medium*. enriched with 2.5 mg/l of zeatin Bar: a = 1.47 cm; b = 1.5 cm; c = 1.44 cm.

4.3.7 - *In Vitro* Elongation of subcultured axillary shoots from Stc_s.

It is widely known that cytokinins are responsible for development of the aerial part of the plants and, in particular, the cytokinin trans-zeatin is the most used for "*in vitro*" growth of Olea's cultivated varieties (Rugini, 1990; Grigoriadou *et al.,* 2002). We therefore tested different concentrations of this hormone during the II Stage of *in vitro* micropropagation, corresponding to the elongation of axillary shoots previously induced to vegetative growth. In particular, axillary shoots induced on $ORZ_{0.5}$ medium were excised from mother Stc and sub-cultured for 45 days on the following medium: OR, without hormone, and ORZ_2, $ORZ_{2.5}$, ORZ_3, ORZ_5, enriched with zeatin at the concentrations of 2mg/l, 2.5mg/l, 3mg/l and 5 mg/l, respectively. We will refer to these explanted axillary shoots as axillary shoot cuttings (Asc).

Asc length was monitored each two weeks and the results are illustrated in figures 4.3.7.1 and 4.3.7.2.

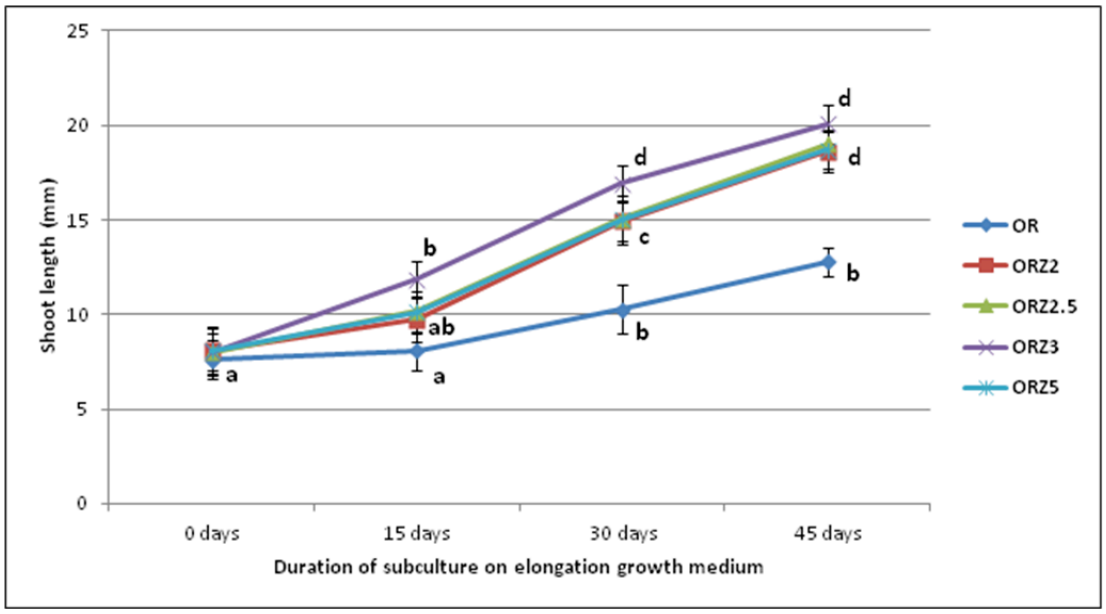

***Figure 4.3.7.1*:** Length of axillary shoot cuttings (Asc) of *Olea europeae* L subsp. *europaea* var. *sylvestris* at different times after subculture on different elongation medium. OR (medium without hormone); ORZ_2 (medium enriched with 2 mg/l of zeatin); $ORZ_{2.5}$ (medium enriched with 2.5 mg/l of zeatin); ORZ_3 (medium enriched with 3 mg/l of zeatin); ORZ_5 (medium enriched with 5 mg/l of

zeatin). The results represent the average value of two independent replicates. For statistical evaluation of data, Analysis of Variance (ANOVA) followed by Turkey test was applied Values marked with different letters show statistically significant differences for $P \leq 0.05$.

A significant lengthening of shoot was observed in all the medium tested (*Fig. 4.3.7.1*). However, in presence of zeatin, shoot lengthening, beside occurring earlier, was significantly greater in zeatin enriched medium (ORZ_2, $ORZ_{2.5}$, ORZ_3, ORZ_5) compared to the medium without hormones (*Figs. 4.3.7.1 and 4.3.7.2*). Within the range of applied concentrations, no significant differences were instead observed between medium with different zeatin concentrations (*Figs. 4.3.7.1 and 4.3.7.2*) .

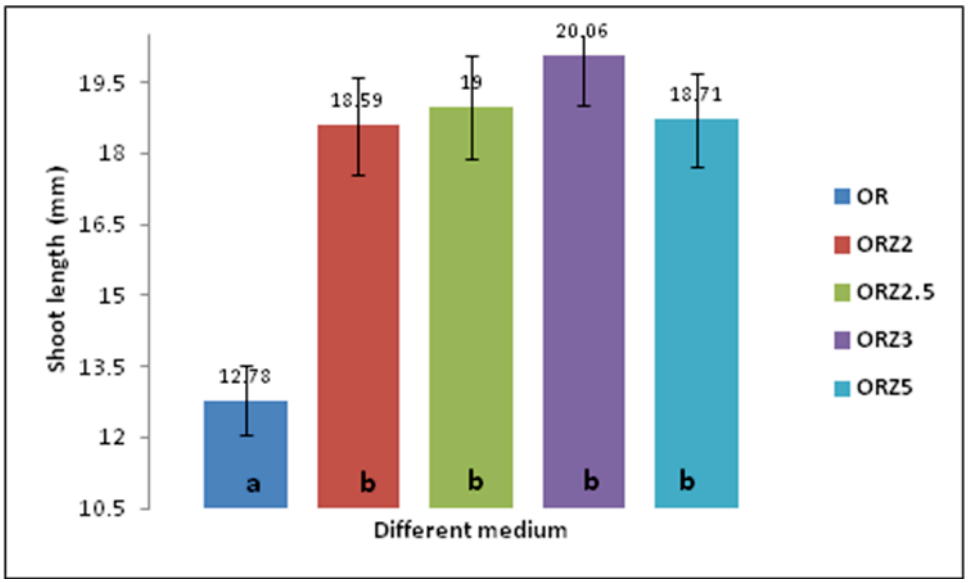

***Figure 4.3.7.2*:** Length of axillary shoot cuttings (Asc) of *Olea europeae* L. subsp. *europaea* var. *sylvestris* after 45 days of subculture on different elongation medium. OR (medium without hormone); ORZ_2 (medium enriched with 2 mg/l of zeatin); $ORZ_{2.5}$ (medium enriched with 2.5 mg/l of zeatin); ORZ_3 (medium enriched with 3 mg/l of zeatin); ORZ_5 (medium enriched with 5 mg/l of zeatin). The results represent the average value of two independent replicates. For statistical evaluation of data, Analysis of Variance (ANOVA) followed by Turkey test was applied Values marked with different letters show statistically significant differences for $P \leq 0.05$

In all the samples, callus formation was always observed at the base of the shoots, even though its dimension was progressively higher on Asc_s growing at

increasing zeatin concentrations (*Fig. 4.3.7.3* b-e) compared to that formed on Asc_s growing on medium deprived of hormone (*Fig. 4.3.7.3* a)

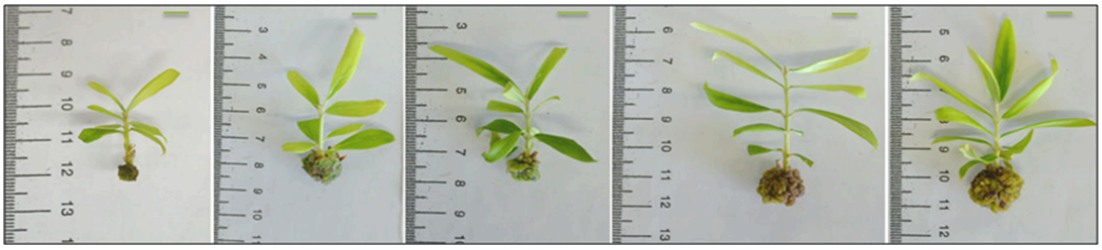

***Figure 4.3.7.3*:** Axillary shoot cuttings of *Olea europeae* L. subsp. *europaea* var. *sylvestris* after 45 day of culture on different elongation medium. Arrow indicates callus formation. OR (medium without hormone); ORZ_2 (medium enriched with 2 mg/l of zeatin); $ORZ_{2.5}$ (medium enriched with 2.5 mg/l of zeatin); ORZ_3 (medium enriched with 3 mg/l of zeatin); ORZ_5 (medium enriched with 5 mg/l of zeatin The results represent the average value of two independent replicates Bar: a = 0.57 cm; b = 0.5 cm; c = 0.6 cm; d = 0.56 cm; e = 0.56 cm.

CHAPTER 5

SECTION B - *In vitro* propagation of *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Hoffm. *et* Link): molecular markers of adventitious root formation.

5.1 BACKGROUND

It is commonly accepted that adventitious root formation includes three different phases (De Klerk *et al.,* 1999). The first, named *induction phase,* is characterized by the recovery of cell proliferation in specific domains of differentiated cells. This is followed by the *initiation phase*, resulting into the anatomical organization of root primordium, which is the prelude to the *protrusion phase,* corresponding to the emergence of primordium (Berthon, 1990; Heloir, 1996).

Certainly, the sequence of molecular, biochemical and morphogenetic events that occur during these three phases involve many hormones and require the activation of specific genetic networks (Benfey *et al.,* 1993; Goldfarb *et al.,* 2003; Sanchez *et al.*, 2007; Ricci *et al.,* 2008; Lucas *et al.,* 2011).

Concerning the *induction phase*, characterized by the progression of cells into cell cycle, genes involved in cell cycle machinery as well as genes encoding either enzymes related to DNA synthesis or histone proteins, necessary for the packaging of newly replicated DNA, are essential component of these networks (Tamas *et al.,* 1992). As far as hormone role is concerned, priority is assigned to auxin as an effective inducer of adventitious root formation in many species (Diaz-Sala *et al.,* 1996; Goldfarb *et al.,* 1998; De Klerk *et al.,* 1999).

5.1.1 - Histones as marker of cell proliferation in adventitious root formation.

As mentioned, the development of adventitious roots implies the recovery of cell proliferation and the activation of a whole series of genes including the genes which encode the different histone fractions. The histone H3 is one of four histone types which form nucleosome core and as such are involved in the packaging of DNA molecule. H3 consists of a globular domain and an unstructured N-terminal tail, which protrudes from the surface of the nucleosome and is involved in the stabilization of chromatin (Arents *et al.,* 1991; Luger *et al.,* 1997; Tse *et* Hansen, 1997; Tse *et al.,* 1998). The N-terminus tail of histone H3 is often subject to phosphorylation and such modification is believed to be involved in the transcriptional activation that requires chromatin decondensation (Cheung *et al.,* 2000; Crosio *et al.,* 2000). Notably, a substantial increase in the expression of the variant *H3-1* gene has been detected after 1 day of treatment with auxin in cell culture of alfalfa *Medicago varia* undergoing to somatic embryogenesis (Tamas *et al.,* 1992). All these results pushed as to consider *H3* gene, as a good marker of DNA replication phase, also in relation to adventitious root morphogenesis.

5.1.2 - Auxin Signaling in adventitious root formation

On the other hand, literature data clearly show that in plant morphogenesis, including adventitious root formation, a very complex cross-talk between auxin, other hormone classes and several genetic circuits is active (Abel *et* Theologis, 1996; Blažková *et al.,* 1997; Caboni *et al.,* 1997). In line with this cross-talk, auxin is able to modify the transcription level of many genes some of which result to be rapidly induced (Abel *et al.,* 1994; Abel *et* Theologis, 1996; Sawa *et al.,* 2002; Pufky *et al.,* 2003; Cluis *et al.,* 2004).

Among the genes which feature as early responsive to auxin, members of the following gene family are included: *SMALL AUXIN-UP RNAs* (*SAUR*s), GH_3-related transcripts, *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*), *AUXIN REPONSE FACTOR* (*ARF*). Therefore, investigating the changes in the expression pattern of

these genes could be important for unraveling molecular mechanisms underlying auxin action.

In my PhD work, I focused the attention on a member of *ARFs* gene family, whose features will be briefly discussed in the following paragraph.

5.1.3 - *ARF* gene family and its cross-talk with auxin

Arabidopsis genome contains 23 *ARF* genes and phylogenetic analysis revealed that the encoded ARF proteins fall into three branches:

- *Class I* which includes most of the members and is commonly subdivided into: subclasses *Ia* with five members; subclasses *Ib* with eight members; and subclasses *Ic* with two members. The middle region of class I members is rich in Pro, Ser, Gly, or Leu; some of these members act as repressors (Ulmasov *et al.,* 1999b; Tiwari *et al.,* 2003).
- *Class II* which includes five members, whose middle region is rich in Glu; some of these members act as activators (Ulmasov *et al.,* 1999a).
- *Class III* which includes three members that are the most divergent compared to those belonging to the other two classes.

The ARF polypeptides vary in size ranging from 57 (ARF13) to 129 kD (ARF7); such variation in size is primarily related to the different amino acid content in the middle region.

ARF proteins are the first identified members of AuxRE-binding protein family (Ulmasov *et al.,* 1997a). At this respect, it must be underlined that many of gene families with auxin-induced expression, share a common sequence, TGTCTC or variants, in their upstream regulatory regions (Ballas *et al.,* 1993 Pufky *et al.,* 2003; Nemhauser *et al.,* 2004). The regions including this sequence are known as *Auxin-Responsive Element* (AuxRE) (Ulmasov *et al.,* 1995, 1997b).

The identification of the AuxRE led to isolate ARF1, the founding member of the AuxRE-binding protein family (Ulmasov *et al.,* 1997a). ARF are short-life nuclear proteins, with specifically bind AuxRE elements in the promoter region of auxin response genes, thus activating or repressing transcription of the genes, depending on the nature of the central domain (Ulmasov *et al.,* 1999b; Tiwari *et al.,*

2003). ARFs can bind tandem repeat AuxRE sequences as homodimers, with other ARFs, or dimers with repressive Aux/IAA proteins (Ulmasov *et al.,* 1997a, 1999b) (*Fig.5.1.1*). Therefore, Aux/IAA_s indirectly regulate transcription of auxin induced genes, through interaction with ARFs, since they do not bind DNA but may affect gene transcription by dimerization with ARFs which repress ARF action (Tiwari *et al.*, 2003).

In figure 5.1.1 it is summarized the auxin signaling pathway leading to the activation of Auxin-responsive genes and its cross-talk with ARF protein. The signaling begins with the binding of auxin to TIR1 subunit of the ubiquitin ligase complex SCF^{TIR1}. The auxin receptor TIR1 possesses a F-box region, with a sequence of repeated leucine that facilitates the protein-protein interactions. The binding of auxin to TIR1 subunit induces a conformational change in the ubiquitin E3 ligase complex SCF^{TIR1}, which in turn activates the E3 ligase, bringing to the ubiquitination of Aux/IAA and its hydrolysis by the proteasome. The ARF proteins in the absence of negative regulators Aux/IAA, stimulate or repress gene expression, according to the specific ARF. Moreover, in many cases, the absence of repressive Aux/IAA increases the dimerization of ARF on AuxRE, resulting in further enhancement of its action (*Fig.5.1.1*) (Taiz *et* Zeiger, 1991a).

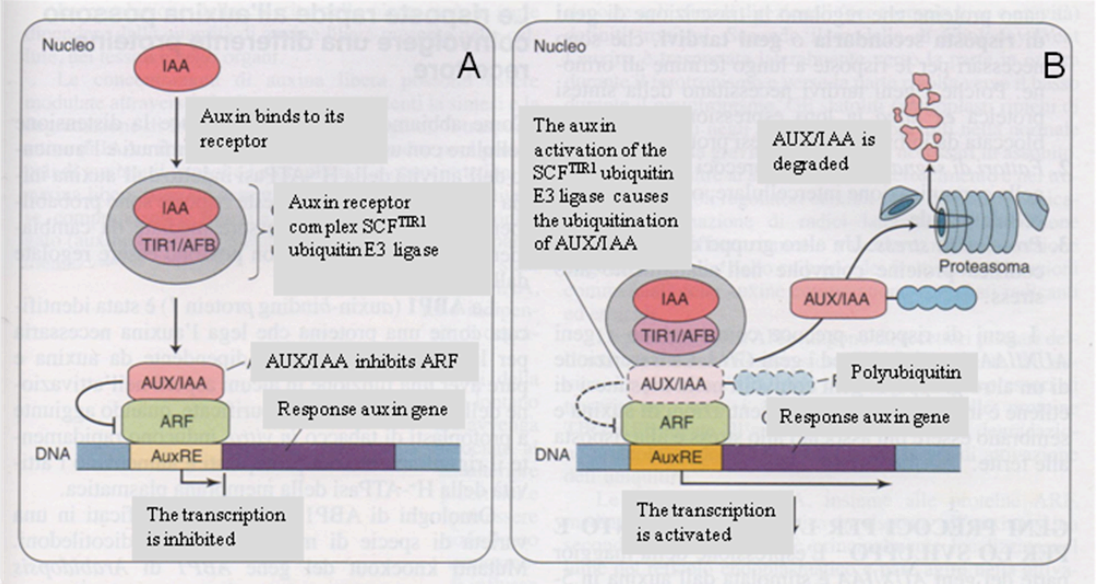

Figure 5.1.1: Model for the binding of auxin with the auxin receptors TIR1/ABF and subsequent transcriptional activation of genes that respond to auxin. **A)** In the absence of auxin, the repressors AUX /IAA inhibit transcription of genes induced by binding to auxin transcriptional activators ARF, relegating in an inactive state. The binding of auxin with the SCF^{TIR1}/ABF complex promotes their association with AUX /IAA protein. **B)** The SCF^{TIR1}/ABF complex activated from auxina, attach the ubiquitin molecules to proteins AUX/IAA, which promote the destruction by the 26S proteasome. The removal and degradation of proteins AUX/IAA, frees the transcriptional activators ARF related to auxin response elements (AuxRE) stimulate the transcription of genes induced by auxins. **C)** In the majority of genes auxin induced, two proteins ARF dimerize on AuxRE, resulting in further stimulation (*'potentiation'*) of gene transcription. Image by Taiz et Zeiger 1991.

On the basis of described events, in order to reach some further insights into the molecular events leading to adventitious root formation from *Oleaster* Smc_s, we decided to investigate the expression level of:

i) a member of *HISTONE* gene family, as a marker of cell replication.

ii) a member of *ARF* gene family, in relation to its involvement in auxin signalling.

5. 2. MATERIALS AND METHODS

5. 2.1 - Plant material

For analyzing the expression levels of *OesARF* and *OesH3* genes, Smc_s (about 5mm high) exhibiting 3 nodes were used.

In particular, transcript levels were evaluated in the basal portion of: i) Smc_s grown under sterile conditions on ORZ_5 medium for vegetative growth (see Section A-*Tab. 4.2.2*), corresponding to the starting point of induction phase; ii) in Smc_s grown under sterile conditions on ORZ_5 medium and then transferred for 4 days to the different rooting conditions (T_1; T_2; T_3; T_4) previously described (Section A – paragraph 4.2.3).

Smc_s basal portions were excised and immediately frozen with liquid nitrogen.

5. 2.2 - RNA isolation and sscDNA synthesis

Total RNA (100 mg) was isolated from basal portions of **Smc_s**, using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) as previously described (Bruno *et al.,* 2009). RNA was resuspended in RNase-free water (50 μL), treated with DNase I (100 μL final volume) at 37°C for 50 min, re-precipitated and concentrated (40 μL). The RNA was measured by the NanoDrop Spectrophotometer ND-1000 and quality was checked by electrophoresis (28S rRNA/18S rRNA ratios). Single-strand cDNA was synthesized from total RNA (3 μg) by the SuperScript III Reverse Trascriptase and the oligo-dT(20) following the manufacturer's instructions (Invitrogen, Milan, Italy).

5. 2.3 - cDNA library generation

The genes investigated were present in cDNA library made available by research group of Prof. M.B. Bitonti. This cDNA library was generated from 50-100 μg of total RNA extracted from leaves of *O. europeae* subsp. *europeae* var. *sylvestris* plants, using SMART system and cloning the sequence (around 1.2kb) in the

pSPORT1 vector. The sequencing analysis was performed from 5'. Generation and sequencing of the library was performed by Eurofins MWG GmbH cDNA Laboratory Fraunhoferstr (De) service.

5. 2.4 - Primer design

The oligonucleotide primer sets used for qRT-PCR analysis were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the strategies set up by Yokoyama and Nishitani (2001). Experimentally optimal primers were identified based upon their ability to meet several standards: (a) robustness: successful amplification over a range of annealing temperatures, (b) specificity: generation of a single significant peak in the melting curve, and (c) consistency highly reproducible of Ct values within the reactions of a triplicate.

The average amplification efficiency of each primer pair was determined, and primers performing poorly were replaced. The average efficiency of all of the primer pairs discussed in this study ranged between 0.95 and 1.0. After checking independent trials of several housekeeping genes, *Histone H2b* produced the most reproducible results across various cDNAs, and was used as a normalization control in according with De Almeida *et al.,* (2010). The primers used for qRT-PCR are listed in Table 5.2.1.

Table 5.2.1: Genes, primers, PCR conditions and products in expression assays

Gene	**Primers 5'–3'**	**Ann. temp. (°C)**	**Base pairs**
OesARF	FW: TGAGACCCAAAAAGGACCAC BW: CTTCCCTCCACTTGGGTTCT	60	137
OesH3	FW: CTACCATTCCAGCGTTTGGT BW: GACCCACAAGGTAAGCCT	60	115
OesH2b	FW: CTCGGGAGATTCAGACTGCT BW: TTCATCAATTCAGGAGCTGGT	60	113

5.2.5 - Quantitative real-time PCR (qRT-PCR) and amplification conditions

Quantitative real-time PCR (qRT-PCR) was performed by STEP ONE (Applied Biosytems). Single colour thermocycler with *Power SYBR ® Green PCR Master Mix 2X* (Applied Biosystem) (Cat. No. 4368702).

Amplification reactions were prepared in a final volume of 20 µL: Power SYBR Green PCR Master Mix (Applied Biosystems) (2X) containing the (AmpliTaq Gold ® DNA Polymerase, LD, SYBR ® Green I, buffer, dNTPs, $MgCl_2$, ROX ™ passive reference dye); dATP-dCTP-dGTP (0.4mM) and dUTP (0.8mM), each primers (0.4 µM); and cDNA (25 ng). All reactions were run in triplicate in 48-well reaction plates, and negative controls were set. The cycling parameters were as follows: one cycle at 95°C for 10 min to activate the enzyme, followed by 40 cycle of denaturation at 95°C for 15 s and annealing-extension at 60°C for 60 s. After reaction, in order to confirm the existence of a unique PCR product the "melting curve" (Lekanne Deprez *et al.,* 2002) was evaluated by an increase of 0.5°C every 10 s, from 60°C to 95°C. We obtained a unique "melting peak" in every reaction and the PCR products were verified by 1% agarose gel electrophoresis.

5.2.6 - Data analysis

The results of real-time PCR were analysed using STEP One Software 2.0 (Applied Biosystems), a program that facilitates the analysis of the kinetics of each performed reaction. Cycle threshold (CT) values were obtained with the STEP One Software 2.0 (Applied Biosystems); software and data were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak *et* Schmittgen, 2001). The means of *OesARF* and *OesH3* expression levels were calculated from three biological repeats, obtained from three independent experiments.

5. 3. RESULTS

5. 3.1 - Isolation and sequence analyses of *O. europeae* subsp. *europeae* var. *sylvestris ARF*- and *H3* -related genes

The genes investigated in the present work were identified from the local Expressed Sequence Tag (EST) database, obtained through the construction of a cDNA library from leaf of *Oleaster* plants.

The cDNA full-length of *OesH3* (*Olea europaea* subsp. *europeae* var. *sylvestris Histone3*) showed an open reading frame of 408 bp; upstream of the ATG triplet it was present the 5'-UTR region of 217 bp, while the 3'-UTR region consisting of 177 bp was present downstream of the stop signal (TGA). The deduced protein was made of 136 amino acids (aa). *In silico* analysis evidenced high homology of *OesH3* with other known homologous genes. The OesH3 protein was blasted in NCBI database and shared the highest identity with that of *Vitis vinifera* (100%), followed by *Arabidopsis thaliana* (100%), *Zea Mays* (99%).

The cDNA full-length of *OesH2b* (*Olea europaea* subsp. *europeae* var. *sylvestris Histone2b*) showed an open reading frame of 441 bp; upstream of the ATG triplet it was present the region 5'-UTR of 115 bp, while the region 3'-UTR consisting of 206 bp. Was present downstream of the stop signal (TGA). The deduced protein was made of 147 amino acids (aa) with a calculated mass of 40 kDa (ExPASy Proteomic Tools, http://www.expasy.org/tools/dna.html). *In silico* analysis evidenced high homology of *OesH2b* with other known homologous genes. The OesH2b protein was blasted in NCBI database and shared the highest identity with that of *Medicago truncatula* (93%), followed by *Arabidopsis thaliana* (89%), and by *Nicotiana tabacum* (88%).

The cDNA full-length of *OesARF* (*Olea europaea* subsp. *europeae* var. *sylvestris Auxin Responsive Factor*) showed an open reading frame (ORF) of 525 bp. The deduced protein was made of 175 amino acids (aa) (ExPASy Proteomic Tools, http://www.expasy.org/tools/dna.html). *In silico* analysis evidenced high homology

of *OesARF* with other known homologous genes. The OesARF protein was blasted in NCBI database and shared the highest identity with that of *Arabidopsis lyrata* (68%), followed by *Arabidopsis thaliana* (63%).

5.3.2 - Expression levels of *OesH3* and *OesARF* in Smc_s under different rooting conditions

The analysis of gene expression in Smc_s of *Olea europaea* subsp. *europaea* var. *sylvestris*: was limited to the induction phase of adventitious rooting process. Therefore, the expression level of both *OesH3* and *OesARF* transcription factor was investigated along a period from 0 to 4 days after placing on rooting medium (see *Tab. 4.2.2*). In particular transcript levels were evaluated, through qRT-PCR: i) in Smc_s grown under sterile conditions on ORZ medium, preliminarily used for inducing vegetative growth (ORZ), corresponding to the starting point of rooting induction phase; ii) in Smc_s grown under sterile conditions on ORZ medium and then transferred for 4 days to the different rooting conditions (T_1- T_4) previously described (Section A - paragraph 4.2.3).

The obtained results revealed that the expression of both *OesH3* and *OesARF* was enhanced by root promoting treatments. Namely, after four days of culture the levels of their transcripts exhibited a significant increase in all rooting conditions compared to ORZ starting point (*Fig.5.3.1*). Moreover, for both analyzed genes the highest expression levels were detected at T_1 and T_2 conditions.

At this respect, it must be underlined that T_2 samples were subjected to long-lasting (overnight) pre-treatment with auxin (IBA) and then transferred to the medium without hormone (T_2). Whereas, under T_1 condition, Smc_s were only transferred from vegetative growth medium (ORZ) to a medium devoid of hormone, without any pre-treatment with auxin (IBA) (*Fig.5.3.1*).

Gene expression was instead progressively lower at increasing IBA concentrations. The observed differences resulted slight for *OesH3* but significant for *OesARF*. All together these results let us to understand that a prolonged intake of IBA at low concentrations enhances the expression of these genes, but also that the

such enhancement is lowered at higher hormone concentration, thus evidencing a feed-back control mechanism. (*Fig.5.3.1*).

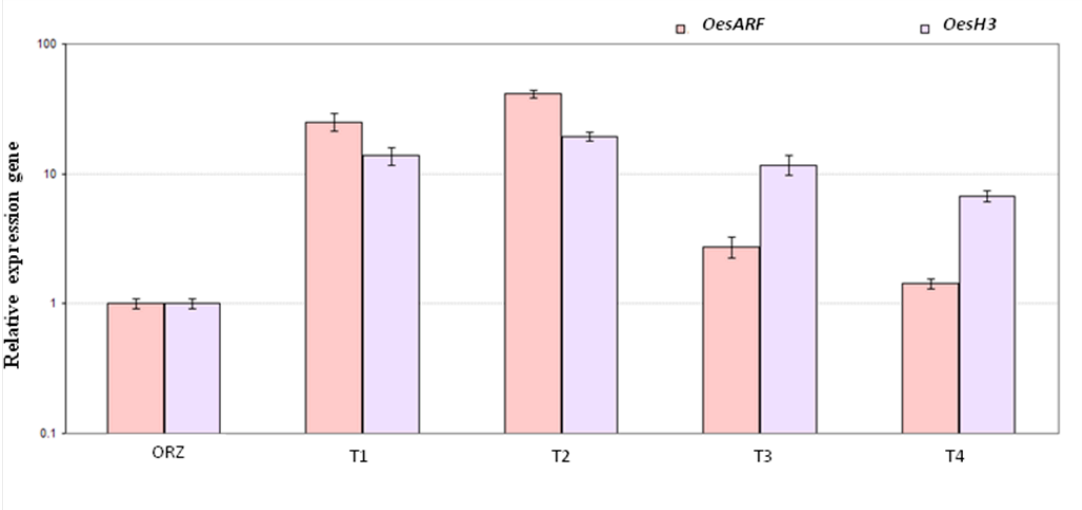

Figure 5.3.1: Levels of *OesARF* and *OesH3* expression in seedling-derived microcuttings of *Olea europaea* subsp. *europaea* var. *sylvestris* grown on vegetative medium (ORZ) and in different rooting media for 4 days. ORZ: OR medium enriched with 5 mg/l of zeatin for vegetative growth; T_1 (no pretreatment + medium deprived of hormones); T_2 (long-lasting pretreatment with 3 mg/l IBA + medium deprived of hormones); T_3 (short-pre-treatment with 3 mg/l IBA + medium enriched with 0.5 mg/l IBA); T_4 (short-pre-treatment with 3 mg/l IBA + medium enriched with 1.0 mg/l IBA). The levels of *OesARF* and *OesH3* were normalized to *H2b* histone gene. Data shown are averages of three biological replicates, with error bars representing SD.

Based on all these results, it appears that the balance between endogenous auxin, which likely accumulate at excision site, and externally supplied IBA can directly or un-directly modulate the level of *OesARF* and *OesH3* transcripts, respectively.

CHAPTER 6

SECTION C - Stress tolerance of *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Hoffm. *et* Link) plants: molecular aspects

6.1 BACKGROUND

6.1.1 - Plant and stress

Stress factor is usually defined as a negative external variable which can determine a potentially disadvantageous effect on the plant. The stresses are divided into biotic stress, if it is induced by other living organisms, or abiotic stress if it is caused by an excess or a deficiency of the chemicals and physical factors that characterize the environment in which the plant lives (Buchanan *et al.,* 2005).

All the biotic and abiotic stresses strongly impact on ecological and agricultural systems, affecting the growth, the development and the distribution of plants (Kosova *et al.,* 2007; Shanker *et* Venkateswarlu, 2011). Drought and cold are among the main stress factors which causes enormous loss to plant productivity, inducing adverse effects on plant vigor and crop yield and endangering the quality of plant production (Kosova *et al.,* 2007; Shanker *et* Venkateswarlu, 2011). Noteworthy, stressful effects of water scarcity are enhanced by it is frequent combination with other types of stresses, like high temperature, high irradiance, salinity (Shanker *et* Venkateswarlu, 2011).

From a physiological point of view, water stress has considerable effects on all major metabolic processes of plant such as: carbon dioxide fixation, respiration, translocation and distribution of metabolites in plant tissues, protein synthesis, energy and lipid metabolism (Mathers, 1999; Shanker *et* Venkateswarlu, 2011). At cellular level, water scarcity affects the degree of turgor which is required for relevant event such as cellular distension and stomatal opening. In addition, water

scarcity causes a progressive damaging of biological membrane integrity, due to the alteration of protein conformation caused by the breaking of hydrogen bonds responsible of protein secondary structure (Mathers, 1999). This in turn compromises the activity of cytoplasmic organelles such as mitochondria, chloroplasts, also altering the photosynthetic apparatus, leading to photo-inhibition and formation of reactive oxygen species (Welling *et al.,* 2006).

Notably, cellular dehydration is established not only by water scarcity or osmotic shock, but also by exposure to low temperature which determines the same harmful effects on the cells previously described, with the most extensive damaging or alteration dealing with membranes (Thomashow, 1999; Close, 1996; Kosova *et al.,* 2008). In fact, a direct and perhaps earliest effect of low temperature is the change in fluidity of cell membranes: fatty acids forming the lipid bilayer of cell membranes at low temperature are responsible of the solidification of membrane in a semi-crystalline state with consequent reduction of fluidity and loss/reduction of activity of associated enzyme (Levitt, 1980; Taiz *et* Zeiger, 1991b; Alonso *et al.,* 1997; Murata *et* Los, 1997, Tomashow, 2010). Thus, the membranes, and especially the plasma membrane, are the primary site of injury induced by both water scarcity and cold stress.

Due to their sessile conditions, higher plants have commonly acquired adaptive mechanisms at the morphological, biochemical and molecular levels that allow them to cope with stressful environmental conditions (Buchanan *et al.,* 2005; Shanker *et* Venkateswarlu, 2011). For example, to overcome the effects of both water scarcity and cold stress osmotic changes are induced in the cytoplasm, related to osmolite accumulation that lowers the aqueous potential of cell compartments, thus preventing either further water loss and the formation of intracellular ice crystals (Taiz *et* Zeiger, 1991b). In addition, cold tolerance also includes the accumulation of cryoprotectives, which lead to a physical and chemical restructuration of cell membranes (Guy, 1990). Therefore with respect to both water and cold stress, chief among adaptive mechanisms is the enhanced expression of a large set of genes, which include those encoding hydrophilic LEA-related proteins (Wood *et* Goldsbrough, 1997; Cellier *et al.,* 1998; Ismail *et al.,* 1999; Zhu *et al.,* 2000; Wisniewski *et al.,* 2002; Basile *et al.,* 2003; Rorat, 2006a; Matteucci *et al.*, 2011).

6.1.2 - The Dehydrin

The Dehydrins (DHNs), which belong to the Group II of the LEA proteins, better known as D-11 subgroup, are the most studied LEA-related proteins (Close *et al*., 1989; Dure *et al.,* 1989; Ceccardi *et al.,* 1994; Close, 1996; Campbell *et al.,* 1998; Ramanjulu *et* Bartles, 2002).

DHNs are thermo stable proteins, able to maintain their integrity in aqueous solutions at up to 100°C. In pure form, DHNs seems to be intrinsically unstructured (Ismail *et al.,* 1999; Lisse *et al.,* 1996), but they may form intrinsic (Borovskii *et al.,* 2000) structures when bound to target molecules (Garay-Arroyo *et al.,* 2000)

DHNs have a wide range of molecular masses, ranging from 9 to 200 kD, (NDong *et al.*, 1997) and, from the structural point of view, are glycine-, lysine- rich proteins while lacking cysteine and tryptophan. Their amino acid composition is characterized by a high content of charged and polar residues which determines their biochemical properties including thermostability. This feature may promote their specific protective function under conditions of cell dehydration, allowing them to prevent coagulation of macromolecules and maintain integrity of cell structure.

Primary structure of DHNs is characterized by the presence of the following conserved domains, defined "*segments*":

a) **K- segment**: it is the most important domain, common to all DHNs and usually located near the C-terminal (Close, 1997). It is a highly conserved lysine-rich motif, consisting of 15 amino acid residues (EKKGIMDKIKEKLPG). However, some single amino acid substitutions and structural modifications may occur within this segment. The K-segment is often present in multiple copies and forms secondary structures where the hydrophilic residues and hydrophobic ones are located on opposite sides of the helix (Close ,1996; 1997). The number of K-segment repeats may vary from 1 to 12 (Close, 1997; Svensson *et al*., 2000). It exhibit α-helix structure; recently it has been proposed that the α-helix structure, forming

intermolecular bundles, is responsible for the amphipathic character of dehydrin (Kosavà, 2007);

b) **S-segment:** it is a domain rich in serine residues and is an important site of a phosphorylation-mediated regulation. Namely, literature data reported that phosphorylated sites promote the interaction of DHNs with specific signal peptides which carries its translocation into the nucleus (Close, 1996; 1997; Campbell *et* Close, 1997)

c) **Y-segment:** it is a **c**onsensus domain, T/VDEYGNP, located near the N-terminus of the protein (Close 1996, 1997). The amino acid sequence of this domain shows similarity to portions of the nucleotide-binding site of chaperones in plants and bacteria but its function is not yet clear.

d) **Φ- segment:** it represents one of less conserved domains, ΦΦE/QXΦKE/QKΦXE/D/Q, usually rich in polar amino acids (Close, 1997).

As previously mentioned, K-segment is the most conserved while the S, Y, and Φ are less conserved.

In pure form, DHNs seems to be intrinsically unstructured (Ismail *et al.,* 1999; Lisse *et al.,* 1996), but they may form intrinsic (Borovskii *et al.,* 2000) structures when bound to target molecules (Garay-Arroyo *et al.,* 2000). The repeated presence of DHN segments and their different arrangements give rise to a variety of conformations (Dure 1993), which led to the identification of amino acid chains with variable length, between 82 and 575 amino acids. The secondary predominant structures of such chains seems to correspond to random windings, formed from amino acids of Φ segment which constitute strongly hydrophobic residues (Dure 1993).

On the basis of the presence of the different segments and their numerical and sequential combination, the DHNs have been subdivided into five subclasses: **YnSKn, YnKn, SKn, Kn** and **KnS** (Close, 1997; Rorat, 2006a).

The precise function of each DHN type *in planta* has not been yet established. To get more insight in the potential roles played by DHNs in plants, their cryoprotective properties, ability to bind lipids and metals, and antioxidative activity have been thoroughly analyzed in *in vitro* studies (Rorat, 2006a). The main question arising from *in vitro* findings is whether each DHN structural type could possess a

specific function and tissue distribution. Recent *in vitro* studies indicated that DHNs belonging to different subclasses exhibit distinct functions (Rorat, 2006a). In particular:

- YSK2-type DHNs bind lipids *in vitro*. At the cellular level, they could act as chaperonins, forming hydrogen bonds with biological macromolecules (Koag *et al.,* 2003). In this way they ensure the formation of hydrophilic interactions necessary for maintaining cell stability under water deficit (Tompa *et* Csermely, 2004). At this respect we may recall that K-segment resembles a lipid-binding class A2 amphipathic α-helical segment, commonly present in proteins that have the ability to bind to acidic phospholipids and vesicles with small diameters. Since this capacity rises from the pronounced α-helicity, the two K-segments present in this class might be involved in membrane binding (Borovskii *et al.,* 2000; Koag *et al.,* 2003).

- KnS-type DHNs have an *in vitro* radical-scavenging activity. Lipid peroxidation is a free radical-mediated degradative process that involves polyunsaturated fatty acids and results in the formation of lipid radicals. Some studies suggested that DHNs belonging to this class facilitates plant cold acclimation by acting as radical-scavenging protein to protect membrane systems under low temperatures (Hara *et al.,* 2003; 2005). KnS-type DHNs also showed metal-binding activity. Thus, these proteins appear to functions also as radical-scavenger, reducing metal toxicity in plant cells under water-stressed conditions. Under conditions leading to the generation of hydroxyl radicals in plants this antioxidative activity may be a crucial function of KnS-type (Kruger *et al.,* 2002).

- SKn- and Kn-type DHNs participate in plant acclimation to low temperature. It has been reported that some DHNs display cryoprotective activity towards freezing-sensitive enzymes. For example the *Citrus unshiu* CuCOR19 DHN was shown to be very effective in protecting catalase and lactate dehydrogenase against freezing inactivation (Hara *et al.,* 2001).

In conclusion, although some functions of DHNs remain unknown, their induction in vegetative tissues under different stresses suggests an involvement in protective actions. According to this action, during water deficit, transcription of *DHN* genes is significantly higher in drought-tolerant than in drought-sensitive species. Correlation between drought tolerance of plants and DHN accumulation was

found in sorghum plants like in sunflowers (Cellier *et al.,* 1998; Wood *et* Goldsbrough, 1997). Also in salt-tolerant lines of rice, the level of ABA-induced *DHN* expression was significantly higher than in sensitive plants (Moons *et al.,* 1995). The involvement of DHNs in the regulation of cell osmotic potential has been showed in various tissues of Arabidopsis and it is possibly related to their synthesis during water and salt stresses (Calestani *et al.,* 1998; Brini *et al.,* 2007). Moreover, a direct interrelationship between the level of DHNs and cold tolerance has been observed (Ismail *et al.,* 1999; Robertson *et al.,* 1994; Wisniewski *et al.,* 2002; Zhu *et al.,* 2000).

For all these reasons DHNs feature as good markers for studying molecular mechanisms involved in stress tolerance by plants. In our work we planned to investigate the expression pattern of one member of this gene family, present in the local Expressed Sequence Tag (EST) database, in plants of *Oleaster* exposed to low temperature. The rational for selecting cold stress was related to:

i) the classification of selected gene, named *OesDHN* (*Olea europaea* subsp. *europaea* var. *sylvestris DEHYDRIN*) as belonging to SK_2-type DHNs, involved in cold response; ii) to the common use of *Oleaster* as root-stook and pollen donor for many olive cultivars. As known olive trees lack dormancy and are sensitive to low temperature (D'Angeli *et al.,* 2003; Matteucci *et al.,* 2011), while oil quality, which is strongly related to lipid and unsatured fatty acid fractions, is improved when plants grow in areas characterized by colder winter. Therefore , for olive breeding program, it should be relevant to dispose of valuable markers for selecting geno/ecotypes of both olive species and its pollen donor with enhanced traits of cold tolerance.

6. 2. MATERIALS AND METHODS

6. 2.1 - Plant material used

For analyzing constitutive *OesDHN* expression, plants (n=3) of *Olea europeae* subsp. *europaea* var. *sylvestris* grown in open field, along Tirrenian coast at Cetraro (CS), Italy were used. Different organs were analyzed: apical vegetative shoot, young (1-2 cm) and adult (6-8 cm) leaves, stem. Samples were excised from plants and immediately frozen for molecular analysis.

For analyzing gene expression under cold conditions, two clones (1 and 2 years old) of *Olea europeae* subsp. *europaea* var. *sylvestris*, obtained through micropropagation of Smc_s (see Section A - paragraph 4.3.3) were used. For each clone, plants (n= 3)were maintained at 4°C in a cold room. Sampling was carried out at different times: at the start of treatment (0h,- Control) and after 30 minutes, 4 hours, 24 hours, 48 hours from the start of the stress condition. For our analyzes only mature leaves (2.5 ± 0.3 cm long) were used, since constitutive analysis evidenced the highest *OesDHN* expression in this mature organ. Samples were excised from plants and immediately frozen for molecular analysis.

6.2.2 - RNA isolation and sscDNA synthesis

Total RNA (100 mg) was isolated from each sample, using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) as previously described (Bruno *et al.,* 2009). RNA was resuspended in RNase-free water (50 µL), treated with DNase I (100 µL final volume) at 37°C for 50 min, re-precipitated and concentrated (40 µL). The RNA was measured by the NanoDrop Spectrophotometer ND-1000 and quality was checked by electrophoresis (28S rRNA/18S rRNA ratios). Single-strand cDNA was synthesized from total RNA (3 µg) by the SuperScript III Reverse Trascriptase and the oligo-dT(20) following the manufacturer's instructions (Invitrogen, Milan, Italy).

6. 2.3 - cDNA library generation

The gene investigated was present in a cDNA library made available by research group of Prof. M.B. Bitonti. This cDNA library was generated from 50-100 μg of total RNA extracted from leaves of *O. europeae* subsp. *europeae* var. *sylvestris* plants using SMART system and cloning the sequence (around 1.2kb) in the pSPORT1 vector. The sequencing analysis was performed from 5'. Generation and sequencing of the library was performed by Eurofins MWG GmbH cDNA Laboratory Fraunhoferstr (De) service.

6. 2.4 - Primer design

The oligonucleotide primer sets used for qRT-PCR analysis were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the strategies set up by Yokoyama and Nishitani (2001). Experimentally optimal primers were identified based upon their ability to meet several standards: (a) robustness: successful amplification over a range of annealing temperatures, (b) specificity: generation of a single significant peak in the melting curve, and (c) consistency highly reproducible of Ct values within the reactions of a triplicate. The primers used for *OesDHN* was:

FWrealtime 5'-AAG GAG AAG CTC CCT GGG TA-3'

BWrealtime 5'-AAA CCA CCA AAG AAG AAA TCA AA-3'.

The average amplification efficiency of each primer pair was determined, and primers performing poorly were replaced. The average efficiency of all of the primer pairs discussed in this study ranged between 0.95 and 1.0. After checking independent trials of several housekeeping genes, 18S rRNA produced the most reproducible results across various cDNAs, and was used as a normalization control. The primer sequence of 18S rRNA was:

FW18S 5'-CAG CCT TCA ATG ATC GGA AT-3'

BW18S 5'-GCG CTG TAA TTT CCT TGC TC-3'.

6.2.5 - Quantitative real-time PCR (qRT-PCR) and amplification conditions

Quantitative real-time PCR (qRT-PCR) was performed on a Bio-Rad Mini Opticon (Bio-Rad, Milan, Italy). Single colour thermocycler with Bio-Rad SYBR Green Supermix (Cat. No.170–8884). Amplification reactions were prepared in a final volume of 25 µL by adding 12.5 µL of the iTaq SYBR-Green Super Mix with ROX (Bio-Rad) containing the (iTaq DNA polymerase 50 units µL-1, 6mm Mg2+, 1mM ROX internal Reference DYE Stabilisers, 0.4mM of dATP dCTP- dGTP and 0.8mM dUTP), 0.4 µM of primers, and 2 µL (25 ng) of cDNA. All reactions were run in triplicate in 48-well reaction plates, and negative controls were set. The cycling parameters were as follows: one cycle at 95°C for 3 min to activate the Taq enzyme, followed by 40 cycle of denaturation at 95°C for 10 s and annealing-extension at 58°C for 30 s. After reaction, in order to confirm the existence of a unique PCR product the "melting curve" (Lekanne Deprez *et al.,* 2002) was evaluated by an increase of 0.5°C every 10 s, from 60°C to 95°C. We obtained a unique "melting peak" in every reaction and the PCR products were verified by 1% agarose gel electrophoresis.

6.2.6 - Data analysis

The data obtained were processed using the program Gene Expression Analysis for iCycler iQ® Real-Time PCR Detection System (v 1.10 - ©2004, Bio-Rad) a program that facilitates the analysis of the kinetics of each performed reaction. Cycle threshold (CT) values were obtained with the Genex software (Bio-Rad) and data were analysed with the $2^{-\Delta\Delta CT}$ method (Livak *et* Schimttgen, 2001). The means of *OesDHN* expression levels were calculated from three biological repeats, obtained from three independent experiments.

6. 3. RESULTS

6.3.1 - Isolation and sequence analyses of *DHN*-related genes from *O. europeae* subsp. *europeae* var. *sylvestris*

The gene investigated in the present work was identified from the local Expressed Sequence Tag (EST) database, obtained through the construction of a cDNA library from leaf of *O. europeae* subsp. *europeae* var. *sylvestris* plants.

The cDNA full-length of *OesDHN* consists of 1243bp and showed an open reading frame of 630 bp. Upstream of the ATG triplet it is present the 5'-UTR region (73 bp), while downstream of the stop signal (TAA) is present the 3'- UTR region (540 bp).

The deduced protein was made of 209 amino acids (aa) (ExPASy Proteomic Tools, http://www.expasy.org/tools/dna.html). The deduced protein includes: a S-Segment between 69 and 77 aa residues; two K-Segments between the aa residues 125-139 et 170-185 respectively, near the C-terminal region. This protein composition supports the idea that the isolated cDNA encodes a SK_2-type Dehydrin. Note that in literature SK_2-type Dehydrins have been found to be preferentially induced by cold condition (Danyluk *et al.,* 1998; Nylander *et al*., 2001; Karlson *et al.,* 2003; Alsheikh *et al.,* 2003; Rorat *et al.,* 2004).

In silico analysis evidenced high homology of *OesDHN* with other known homologous genes. The OesDHN protein was blasted in NCBI database and shared the highest identity with that of *Solanum chilense* (62%), followed by BDN1 in *Paraboea crassifolia* (59%), by DH3 in *Coffea canephora* (55%) and finally with DHN in *Citrus x paradise* (50%).

6.3.2 - *OesDHN* Constitutive Expression

In our analysis we first proceeded to define the pattern of gene expression in the various organs of the plant. As reported in the figure 6.3.1, the gene is expressed

in all analyzed organs, but at different levels; in particular, in agreement with literature, *OesDHN* transcripts were found particularly abundant in the leaves and in the stem. It is interesting also to note that the expression of this gene in the leaf is modulated in relation to the stage of development (Fig 6.3.1.)

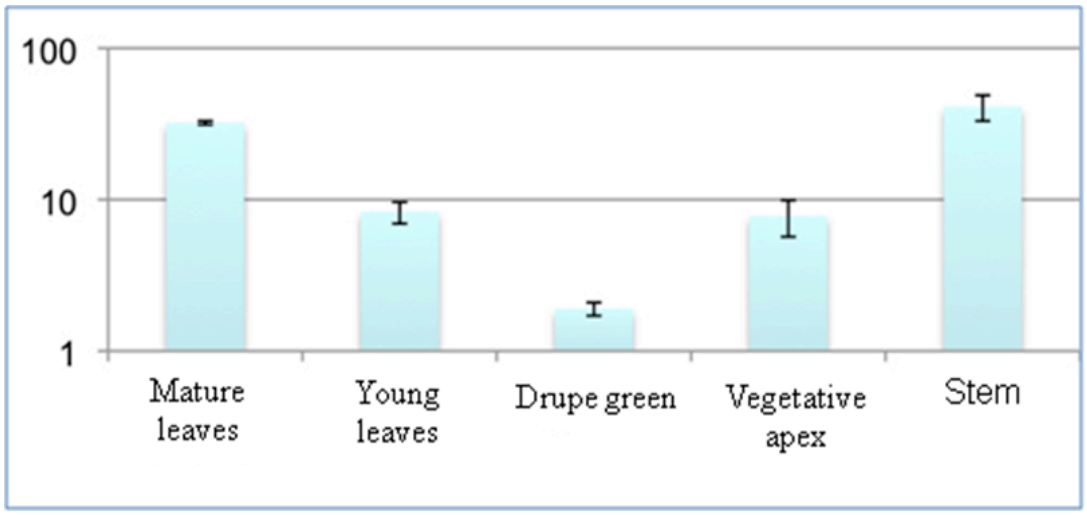

Figure 6.3.1: Relative Expression levels of *OesDHN*, estimated by qRT-PCR, in different organs of *Olea europaea* L. subsp. *europaea* var. *sylvestris* grown in open field. Data are average values of three biological replicates, with error bars representing SD.

6.3.3 - *OesDHN Expression pattern under cold stress condition*

In order to test the putative involvement of SK_2-type *OesDHN* in the response of *Olea europaea* L. subsp. *europaea* var. *sylvestris* plants to stress, gene expression level was evaluated in the mature leaves (length 2.5 ± 0.3 cm) of young plants (1-2 years old) exposed to low temperatures (4°C) for different time intervals (0h-Control; 30'; 4h; 24h; 48h).

Firstly, the obtained results (*Fig 6.3.2*) revealed a significant enhancement of *OesDHN* expression following exposure to low temperatures in the leaves of both 1 and 2 years old plants. However, timing and rate of such increase, clearly differed depending on the age of the plant. More in particular, in one year old plants, after only 30 minutes of treatment the level of gene expression was 10 folds higher compared to the control and did not change any more with the prolonging of

treatment (*Fig 6.3.2*). In two years old plants, gene expression level no much increased until 4h of treatment, but it was 10 and 100 folds higher in exposed than in control plants after 24 and 48 h of cold exposure, respectively (*Fig 6.3.2*).

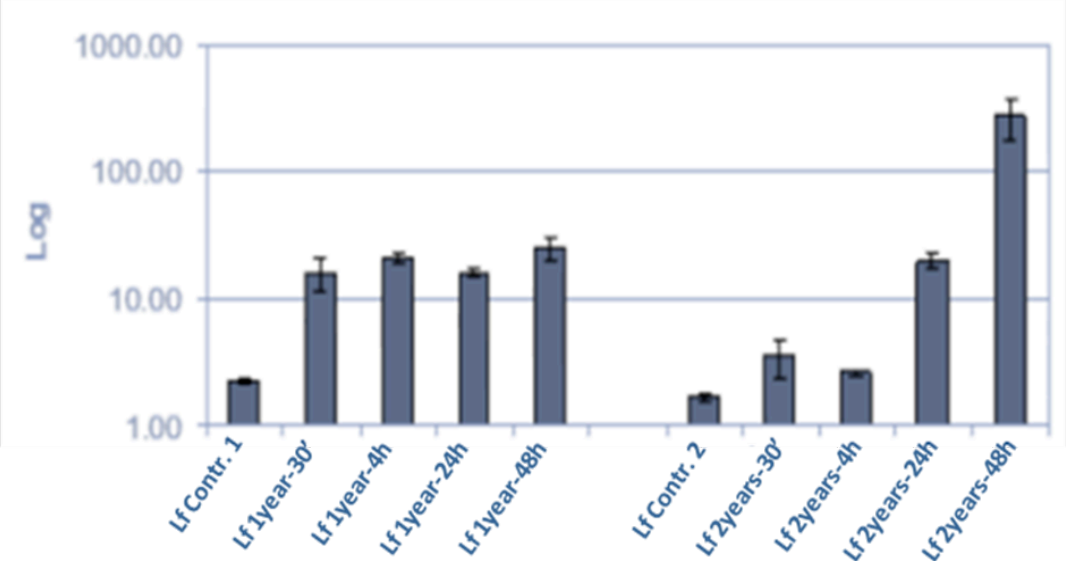

Figure 6.3.2: Expression relative levels of *OesDHN*, estimated by qRT-PCR, in leaves of one and two years old plants exposed to cold (4 ° C) for different times. Lf = leaf. Contr.1 and 2= un-exposed plants. Data are average values of three biological replicates, with error bars representing SD.

CHAPTER 7

GENERAL DISCUSSION

As largely mentioned in the previous chapter, *Oleaster* (*Olea europaea* L. subsp. *europaea* var. *sylvestris*) is the wild form of cultivated olive and represents a prevalent component of plant formation named Mediterranean "*Macchia*". *Oleaster* potentiality is related on one hand to its use as root-stook and pollen donor for many olive cultivars, on the other hand to its aptitude to be used for a rapid re-implantation of vegetation on eroded soil, because of a dept root system and high tolerance to extreme environmental conditions (i.e. water scarcity, salinity, high irradiance, nutrient deficiency).

To overcome some difficulties in developing such *Oleaster* potentiality, the present PhD work was, first of all, addressed to set-up an efficient procedure for its micropropagation through *in vitro* culture of stem cuttings, while searching for early molecular markers of adventitious rooting. At this aim, we used microcuttings derived from *in vitro* germinated seedlings (Smc_s) and microcuttings excised from plants grown in open fields (Stc_s). In the case of Smc_s, the main handicap was related to the poor capacity of *Oleaster* seeds to undergo germination, which, at the beginning of our work, was found to be about 46% after 60gg. We were able to optimize the condition for germination through seed scarification and vernalization treatment, which allowed us to obtain by far a higher germination percentage (68%) and above all to shorten the period (15days) for germination to occur (these results have been already published: Chiappetta *et al.,* 2010). In the case of Stc_s, the major risk was the contamination of explanted microcuttings and we were able to implant sterile cultures in about 96% of implanted Stc_s.

The second step of microcutting *in vitro* culture is related to the recovery of vegetative growth (*bud induction phase*) and axillary shoot elongation (*elongation phase*). During this step, we tested the efficiency of two mediums, MSZ and ORZ, widely used in literature for *in vitro* propagation of *Olea europaea* species and the major focus was on discovering cytokinin optimal concentrations (Rugini, 1984; Mencuccini *et al.,* 1997; Rokba *et al.,* 2000). Applying different zeatin

concentrations to Smc_s and Stc_s (2.5 mg/l and 0.5 mg/l of zeatin, respectively), it was possible to achieve a good balance between vegetative growth (estimated as: *budding percentage*; *shooting percentage*; and *shoot length*) and un-desirable callus formation at the microcuttings basal end. Therefore a good stabilization of Smc_s and Stc_s culture was obtained.

For woody plants, rooting phase constitutes a critical step of *in vitro* micro-propagation process. It is known that auxin plays a major role in root organogenesis (Abel *et* Theologis, 1996; Blažková *et al,* 1997; Caboni *et al,* 1997). Therefore, we tested different concentrations of this hormone (IBA) which was supplied by different procedures. It was evident that the procedure, besides concentration and duration of auxin supply, significantly influenced the formation of adventitious roots from explanted microcuttings. Namely, in Smc_s a higher rooting percentage, associated to a longer root length, was obtained after a short pre-treatment with IBA followed by culture in 1 mg / l IBA medium (T_4), compared to both the total absence (T_1) and lower concentration (T_3) of hormone in the medium as well as to a long lasting pretreatment (T_2).

By analyzing, at the histological level, the adventitious roots formed at T_4 condition, a tissue organization and patterning quite comparable to that of embryonic roots was observed. The only differences that we observed dealt with the presence of a smaller root cap and a premature protoderm differentiation with abundant root hairs starting from elongation zone. Both these features are consistent with the variations in the root hormonal network induced by *in vitro* cultures (Harry *et* Thorpe, 1999; Abousalim *et al.,* 2005; Woodward *et* Bartel, 2005). At this respect we may recall that auxin seems to have effects on elongation rather than production of root hairs (James *et al.,* 1996; Clark *et al.,* 1999; Schiefelben *et al.,* 2000). However, auxin is also known to increase the production of ethylene in the roots (Abeles *et al.,* 1992). Notably, ectopic root hairs formation has been detected in ethylene-mutants of *Arabidopsis* (Masucci *et* Schiefelbein, 1994; Tanimoto *et al.,* 1995). On the other hand, it has been demonstrated that in *Arabidopsis* seedlings growing in presence of an inhibitor of ethylene synthesis, the addition of IAA allowed the normal formation of root hairs likely by inducing ethylene production (James *et al.*, 1996). Therefore,

the abundance of hairs along the adventitious roots of T_4 microcuttings grown in presence of auxin may include these mechanisms.

Note that the correct developmental pattern of adventitious root system of T_4 microcuttings was further supported by their successfully overcoming acclimatation phase in pots. At this respect we may recall that T_2 pretreatment was, instead, able to induce the highest number of roots for cutting, but these roots reached the lowest length compared to those developed under the other culture conditions. Such reduced root development was consistent with the un-ability of T_2-rooted Smc_s to overcome acclimatization stage. It must be also recalled that, under the same experimental conditions, rooting of Stc_s resulted entirely inadequate, since less than 3% of implanted microcuttings produced adventitious roots. In line with literature data, is likely that a different endogenous hormone level and homeostasis present in adult plants vs young seedlings of *Oleaster* could account for the different rooting performance of Stc_s compared to Smc_s (Abousalim *et al.,* 2005).

Studies were also carried out on the transcriptional events underlying the induction phase of adventitious rooting process, with the aim to enlarge knowledge in this field and identify some potential marker for selecting optimal, inductive conditions for *in vitro* culture. The candidate genes that we selected for this aim belonged to H3 and ARF gene families as respective marker of cell cycle progression and auxin signaling. Indeed, both these inter-playing events are essential for the resumption of cell proliferation during the induction phase of adventitious rooting process. According to their putative role, the expression level of both *OesH3* and *OesARF* increased during the induction phase. Moreover, the highest gene over-expression was detected at T_2 condition, leading to the highest number of roots for cutting. However, taking into account that T_2 rooted-plantlets also showed the most reduced root length, which in turns caused plantlets to do not survive, a threshold in the expression level of selected genes seems to be essential for assuring a good performance of rooting process.

Finally, in the last part of the work, we started a study on the molecular basis of *Oleaster* tolerance to stressful conditions. A SK_2-type *OesDHN* was present within the local Expressed Sequence Tag (EST) database made available by Prof. M.B. Bitonti's research group. Taking into account the involvement of SK_2-type

DHNs in cold tolerance, besides water scarcity, the expression pattern of *OesDHN* was investigated in plants of *Oleaster* grown in open field as well as in young plants exposed at low temperature (4° C) under laboratory conditions. A differential level of *OesDHN* transcripts was detected in different organs of plants, with higher levels in mature leaves and stems. Moreover such expression was early and strongly enhanced by cold treatment.

Literature data reported that DHNs, accumulate in different organs of plant during normal vegetative growth and strongly increase under different stresses (Moons *et al.,* 1995). It is largely suggested that this accumulation may correlate with their involvement in avoiding damage produced from dehydration, acting as membranes stabilizer, as well as to their cryoprotective properties, ability to bind lipid and methals, antioxidant activity (Close, 1996; Rorat, 2006a; Kosova *et al*., 2008). At the cellular level, cold exposure determines an undirect cell dehydration and loss of membrane fluidity acting on lipid bilayer of cell membranes. Consequently, the early modulation of *OesDHN* expression following exposure to low temperatures (4 ° C) in *Oleaster* plants can be considered an adaptive response of plant to the induced stress.

CONCLUDING REMARK

In conclusion, we can assert that the propagation of *Oleaster* by seedling-derived microcuttings is feasible with satisfactory results. The analyzed genes *OesARF* and *OesH3* have been found to be, even in a woody species as *Oleaster*, excellent markers of the induction phase along the adventitious rooting process. Therefore, they appear to be suitable: i) to preliminarily and rapidly test and select, additional hormone combinations aimed to further improve rooting and micropropagation efficiency of seedling-derived microcuttings; ii) to be especially helpful for identifying optimal *in vitro* culture conditions for a successfully rooting and propagation of plant-derived stem cuttings.

Concerning the study on the expression pattern of *OesDHN*, its early and enhanced response, at the transcriptional level, under low temperatures, features this gene as a putative functional marker of plant stress tolerance. Therefore, through the analysis of potential polymorphisms, it could be successfully used to select geno/ecotypes with traits of particular cold resistance. Since *Oleaster* is used as root rootstock and pollen donor for many cultivar, this results appears of potential interest for molecular assisted breeding of *Olea* species, which exhibits a mild-tolerance to cold, despite the quality of its products improves under these environmental conditions.

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Publications

Chiappetta A., Gagliardi C., Tripepi M., Bernardo L., Bitonti M. B. (2010) Propagazione "*in vitro*" di *Olea europaea* L subsp. *oleaster*: ricorso a microtalee da semenzali. *Informatore Botanico Italiano* (42): pp.145-149.

Chiappetta A., Gagliardi C., Iaria D., Bruno L., Bitonti M.B. (2012) Expression of *ARF* and *H3* genes in olive cuttings as markers of adventitious rhizogenesis. (under submission to *Tree Physiology*)

Meetings

Bruno L., Gagliardi C., Iaria D.L., Chiappetta A., Bitonti M.B. Pattern di espressione di un gene codificante per una deidrina di tipo SK_2 in piante di *Olea* sottoposte a condizione di stress da freddo. 105° Congresso della Società Botanica Italiana. Milano 25-28 Agosto 2010 (Poster).

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