



**FACULTY OF AGRICULTURE
VITERBO – ITALY**

**PhD RESEARCH IN HORTICULTURE
CYCLE XXI**

Scientific-disciplinary field: AGR-03

DISSERTATION

Presented for the title of
Doctor of Philosophy in Horticulture (PhD in Horticulture)

**THE EFFECT OF ARBUSCULAR
MYCORRHIZA ON MICROPROPAGATED
OLIVE (*Olea europaea* L.) PLANT GROWTH**

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DOTTORATO DI RICERCA IN
ORTOFLOROFRUTTICOLTURA
XXI CICLO

Settore scientifico – disciplinare : AGR-03

**L'EFFETO DELLE MICORRIZE ARBUSCOLARI
SULLA CRESCITA DI PIANTE DI OLIVO
(*Olea europaea* L.) MICROPROPAGATE**

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FINAL EXAMINATION AND DISCUSSION
PhD Research in Horticulture

Aula Blu, 27 February 2009
Faculty of Agriculture, Università degli Studi della Tuscia

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DEDICATION

*Untuk Mama dan Papa tersayang atas cinta dan doanya
For my parents for their never-ending support and love*

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INTRODUCTION

1.1. History, Origin and Botany of Olive

The olive (*Olea europaea* L.) is one of the most ancient domesticated fruit trees and the most extensively cultivated fruit crop in the world, covering an area of about 7.5 million hectares (Fabbri 2009). The olive is native of the Mediterranean region, tropical and central Asia and various parts of Africa. *O. europaea* may have been cultivated independently in two places, Crete and Syria. Archaeological evidences suggest that olives were being grown in Crete as long ago as 2,500 B.C. From Crete and Syria, olives spread to Greece, Rome and other parts of the Mediterranean area (Figure.1). Olives are also commercially cultivated in California, Australia and South Africa.

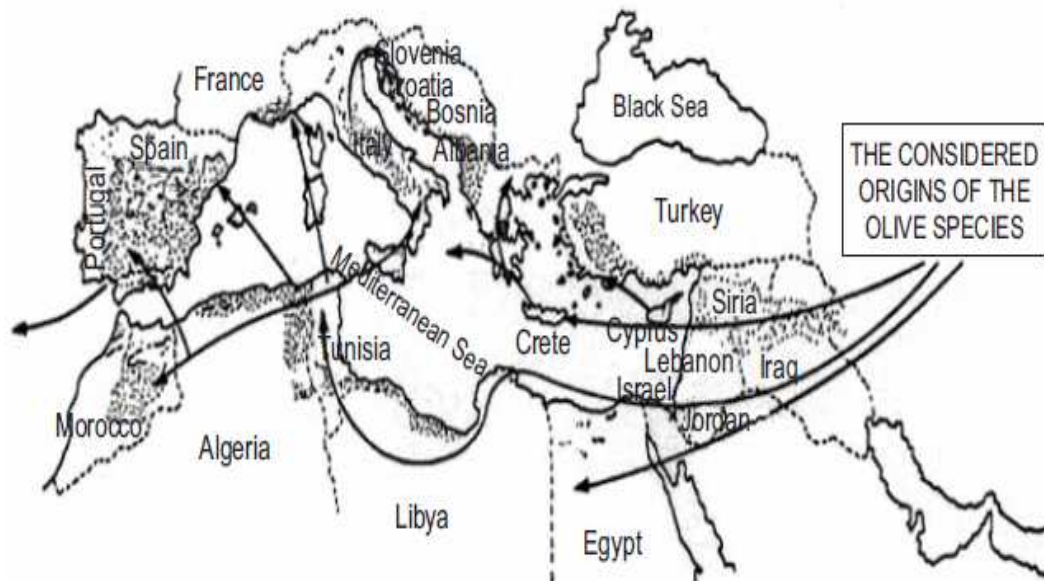


Figure 1.1: Evolution through the millenniums of olive-tree production in the Near East and the Mediterranean basin (Fontanazza 2005).

The true genetic origin of today's cultivated olive, *Olea europaea* L., is not known. Some scientists believe that the "European" olive is a hybrid between two

or more distinct species (Vossen 2007). Other scientists consider the genus *Olea* and species *europaea* to represent just one group of widely diverse plants with “ecotypes” or “subspecies” that are located in different geographic areas. In almost every location where cultivated olives grow, wild olive trees and shrubbery called *oleaster* or *acebuche* also exist. These plants may be seedlings of cultivated varieties spread by birds and other wildlife feeding on the fruit, or they could be more native forms of subspecies or ecotypes that already existed there before the introduction of the cultivated olive. All the species belonging to the *Olea* genus have the same chromosome number ($2n = 46$), and crosses between many of them have been successful. Most scientists now use the nomenclature of *Olea europaea* L. *sativa* to distinguish it from the wild olive subspecies *oleaster* (Lavee 1996).

Classification of olive:

Kingdom: Plantae

Division: Magnoliophyta-flowering plants

Class: Magnoliopsida-dicotyledons

Order: Lamiales

Family: Oleaceae

Genus: *Olea*

Species: *O. europaea*

The olive tree has a wide range of adaptability: the wood resists decay, and when the top of the tree is killed by mechanical damage or environmental extremes, new growth arises from the root system. Whether propagated by seed or cuttings, the root system generally is shallow, spreading to 0.9 or 1.2 m even in deep soils. The above-ground portion of the olive tree is recognizable by the dense assembly of limbs, the short internodes, and the compact nature of the foliage. If unpruned, olives develop multiple branches with cascading limbs. The leaves are thick, leathery, and oppositely arranged; each leaf grows over a 2-year period. Leaves have stomata on their lower surfaces only. Stomata are nestled in peltate trichomes that restrict water loss and make the olive relatively resistant to drought (Martin 2009).

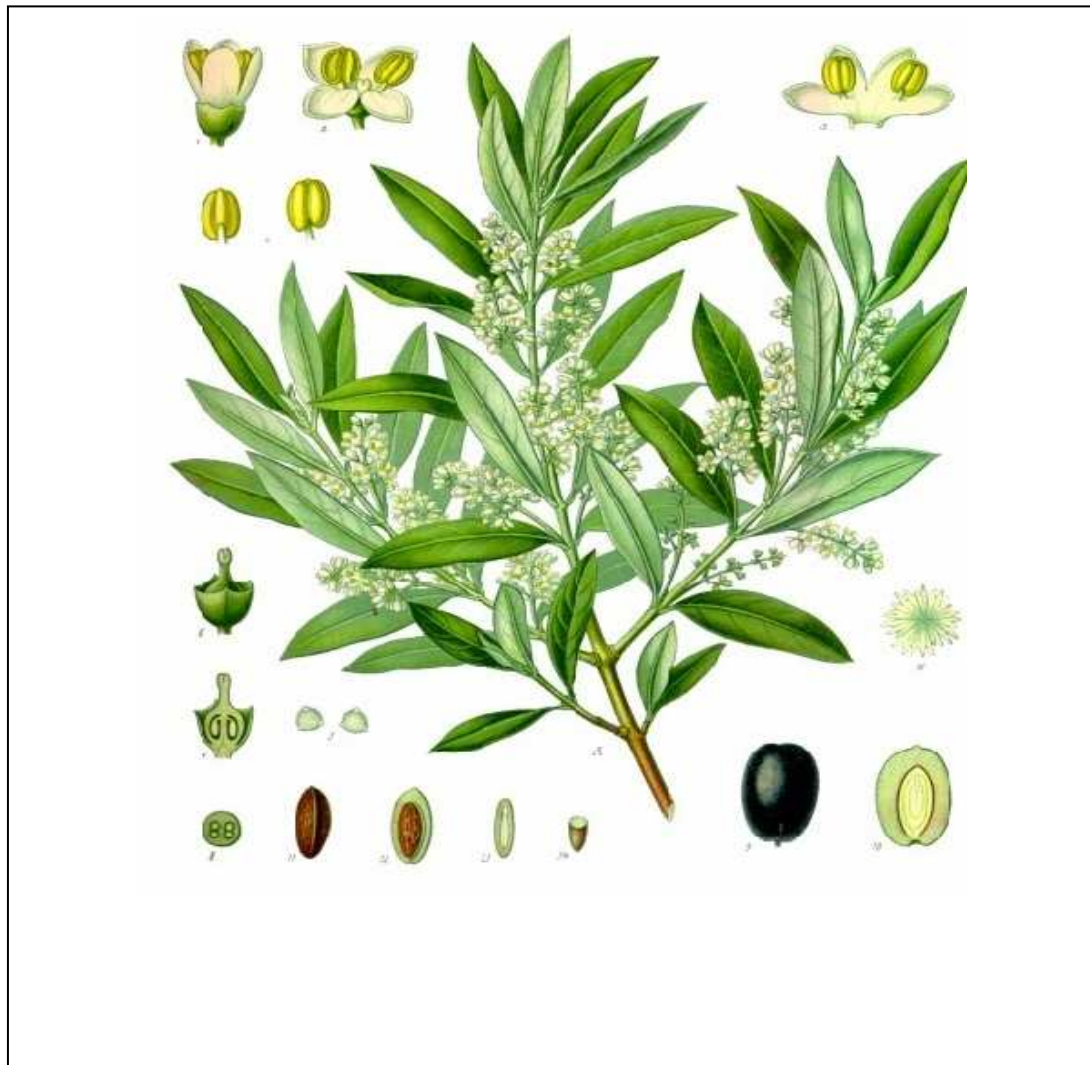


Figure 1.2: 19th century illustration of olive (Koehler 1887)

The flowers are born on the inflorescence and are small, yellow-white, and inconspicuous. Each contains a short, 4-segmented calyx and a short-tube corolla containing 4 lobes. The 2 stamens are opposite on either side of the 2-loculed ovary that bears a short style and capitate stigma. Two types of flowers are present each season: perfect flowers, containing stamen and pistil, and staminate flowers, containing aborted pistils and functional stamens. The proportion of perfect and staminate flowers varies with inflorescence, cultivar, and year. Large commercial

crops occur when 1 or 2 perfect flowers are present among the 15 to 30 flowers per inflorescence. As a rule, more staminate flowers than pistillate flowers are present. The perfect flower is evidenced by its large pistil, which nearly fills the space within the floral tube. The pistil is green when immature and deep green when open at full bloom. Staminate flower pistils are tiny, barely rising above the floral tube base. The style is small and brown, greenish white, or white, and the stigma is large and plumose as it is in a functioning pistil.

The olive fruit is a drupe, botanically similar to almond, apricot, cherry, nectarine, peach, and plum fruits. The olive fruit consists of carpel, and the wall of the ovary has both fleshy and dry portions. The skin (exocarp) is free of hairs and contains stomata. The flesh (mesocarp) is the tissue eaten, and the pit (endocarp) encloses the seed (Martin, 2009).

Moraiolo is Tuscany cultivar, very popular in Italy and other Mediterranean countries (http://www.pruneti.it/olio/moraiolo/moraiolo_cultivar.html), also known as Ruzzolino, Morinello, Morellino, Morello, Oriolo, Corniolo, Cimignolo, Fosco, Nostrale, Assisano, Anerina, Bucino and Carboncella. The trees have medium-to-low vigour with branches having a rising, spreading habit. The crown is gathered and has leaves, which are elliptical-lanceolate in shape, of medium dimension and gray-dark green colour. The fruit is rather small (1.5 to 2 g), rounded and spheroid in shape. Fully mature, it is purple-black in colour, but at the correct picking time it is generally purple-green. This variety has a relatively high and constant fruit production. It is self-sterile and requires specific pollinators, which are Pendolino and Maurino. It resists salty winds. The olive oil content in the fruit is on average between 17 and 20%, but can often be much higher. It has usually low acidity and high quantity of polyphenols. The oil is highly regarded, generally fruity with a slightly bitter aftertaste. Moraiolo is considered a rustic variety, ideal for planting in hilly zones subject to winds. Because of the small size of its fruits together with its tightly attached peduncles this cultivar is not suitable for mechanical harvest. Sensitive to cold and peacock spot (*Cycloconium oleaginum*).

Canino cultivar is especially widespread in Viterbo province area, although also present in other provinces. The olive oil from this cultivar has fruity flavour balanced between pleasantly bitter and pungent with pale green colour, and rather

fluid. The plant is very strong, with foliage relatively dense. The rooting adventitious is good. Leaves are medium sized, elliptical-lanceolate, light green in colour. The inflorescences are small, with 13-14 flowers, and in many cases, it has a high ovary abortion percentage (40%). The drupes have spheroid shape and they are very small (1-1.5 g), maturation is delayed and yield in oil is medium-high (18-20%). The result can be observed on a number of small Lenticels. The cultivar is auto sterile, therefore, it requires appropriate pollinators, such as Rocket, Frantoio, Crucible, Fosco, Leccino, and Moraiolo. The production is good, relatively constant, and amounts to over 20,000 tons of olives. The plant has a good tolerance to cold and flies, while it is sensitive to peacock spot (*Cycloconium oleaginum*) (Lombardo 2003)

Transformation techniques have been developed to olive plant, by using somatic embryogenesis, and transgenic plants, with some desirable agronomic traits, have already been generated in one cultivar. At present, field trials approved by the Italian Health Minister are conducted on transgenic *rolABC*, and *osmotin* plants. Transgenic olive plants, with *rolABC* genes expected plants compact vegetative habitus, smaller number of flowers per plant, and high rooting ability of cuttings. In plants over-expressing *osmotin* gene, higher tolerance to some fungi is expected (Rugini and Gutiérrez Pesce 2006).

1.2 Social and economic importance of olive

The traditional area of olive cultivation and production is the Mediterranean basin, which accounts for 90% of the olive orchards of the world, mainly in Spain, Italy, Portugal, Greece, Turkey, Tunisia and Morocco (Figure 1. 3) and almost 94% of the world's olive oil are produced (IOOC 2008).

The most important reason for olive cultivation is the production of oil and table olives, although the species have other important functions, like the traditional landscape, typical of religious sites, it's suitable in difficult areas such as calcareous, rocky and sloping soils. The importance was also due to other uses, such as lamp fuel, wool treatment, medicine and cosmetic, and soap production (Fabbri *et al.* 2009). Finally, its wood is of great value due to its natural durability and beauty;

hence, in some areas (like Southern Italy), it represents a secondary source of profit from olive orchards (Lambardi and Rugini 2003).

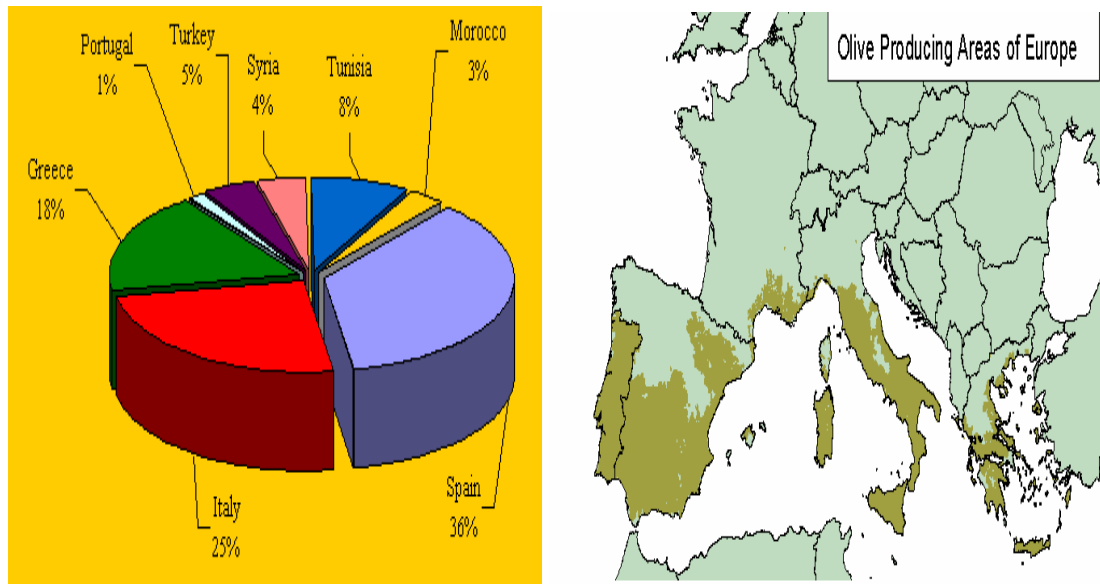


Figure. 1.3: Main producing countries in 2005 (UNCTAD) left; Olive Producing Area of Europe (Peedell *et al.* 2009) right.

In 2005, Italian olive production covered approximately 1.700.000 ha, 80% located in southern Italy. Puglia represents the most important region for the olive production, followed by the Calabria and Sicily. These three regions account for more than 60% of Italian olive production. In terms of olive-oil production, Italy ranks second in the world (after Spain), producing an average oil quantity over the last four years of 550 000 tonnes, mainly represented by extra-virgin and virgin olive oils. As regards olive-oil consumption, Italy is the world leader with a consumption of 650 000 tonnes, corresponding to about 12 kg per head of population (Fontanazza 2005).

The exportations of Italian olive-oil are directed towards different countries, mainly the United States of America, Japan, Canada and Australia, where the oil imported from Italy has gained a strong position in recent years in comparison with oil imported from Spain, Greece and Tunisia.

1.3 Propagation of Olive

Olives are multiplied by seed to obtain rootstock material, or for breeding to obtain new cultivars or new rootstock genotypes with deeper and stronger root systems. It has been proven that root systems grown from seeds are stronger and more consistent than root systems derived from rooting (Faiello 2009). Propagation of olive trees by seed is very frustrating, because the juvenile non-bearing phase is long (10–15 years) and the progeny very often do not even resemble the original mother tree (Vossen 2007).

Although in the past traditional propagation largely utilized a pool of rootable organs and natural formations such as stump suckers, 2- or more-year old portions of branches), at present the olive is almost entirely reproduced by cuttings and graftings (Fabbri *et al.* 2004). Leafy cuttings are obtained from one-year-old vigorous shoots. Cuttings (about 15 cm long, and provided with 2-3 pair of leaves in their upper part) are treated with ethanol solutions or talcum dispersions of indol-3-butyric acid (IBA), prior to being placed in a bottom-heated bench under mist conditions. Grafting propagation is a technique nowadays confined to specific areas (mainly Central and Southern Italy) where, traditionally, many olive farmers still prefer grafted to self-rooted plants when establishing olive orchards, particularly in shallow soils (Lambardi and Rugini 2003). The most common procedure makes use of scions (4-5 cm long) from one-year old branches, which, in spring, are bark grafted on potted or in-field growing olive seedlings.

In spite of recent advances in nursery technology, several problems still affect conventional vegetative propagation of olive, such: the olive cuttings root properly only if collected in two specific periods of the growing season, i.e., before blossoming (spring), and at the onset of autumn growth; adventitious rooting capacity varies greatly among cultivars; olive cultivars are made up of a combination of clones (“cultivar-populations”); hence, they can perform very differently in different nurseries, even when all the physiological, agronomic and propagation conditions are similar; and grafting propagation has been largely abandoned (except in Italian nurseries) because of the high costs of management and to the lack of specific clonal rootstocks (Lambardi and Rugini 2003).

1.4. In vitro propagation

As a consequence of the condition on *in vivo* propagation, the development of micropropagation procedures give much hope to modernize and to improve propagation technology, to standardize the characteristics of nursery olive plants, and to enhance their performance when in field, this technique also allows high quality production and rapid growth of the plants, which are pathogen free on the surface and in the vascular system.

Moreover, *in vitro* techniques allow (i) rapid propagation of cultivars which are difficult to reproduce by traditional propagation, (ii) production of disease-free plants, (iii) application of bioengineering methods, and (iv) conservation of valuable germplasm. Micropropagation of olive has already been reviewed in the past (Rugini 1984; Rugini 1987; Rugini and Fedeli 1990)

In the olive, it is possible to produce plants from either zygotic and mature tissues from different cultivars under the three micropropagation techniques: a) by axillary buds stimulation, b) by organogenesis, and c) by somatic embryogenesis. (Rugini and Gutiérrez-Pesce 2006).

Protocols for *in vitro* propagation of the olive cultivars were reported many years ago and the development of a specific olive medium was formulated and analysis of the main mineral elements of shoot apices from field plants, during their rapid growth (Rugini 1984). Several problems impeded the development of effective protocols of micropropagation, among which: (i) the heavy oxidation of tissues when explants (shoot tips, meristems) were collected from in-field or greenhouse plants, (ii) the difficulty of getting sterile shoots when nodal explants were used, (iii) the laboriousness of establishing shoot cultures with some cultivars. Over the last decade, many advances have been made towards the solution of these problems and the optimization of the various steps involved in olive micropropagation (Lambardi and Rugini 2003). However, today many cultivars have been established and propagated *in vitro* also per commercial purposes.

1.5. Micropropagation and Mycorrhization

Tissue culture is one of the most important applications of modern biotechnology in plant and has been extensively used for the rapid multiplication of

many plant species. Micropropagation techniques allow rapid production of high quality, disease free (Raaman and Patharajan 2006) and uniform planting material in relatively short periods of time. It offers several distinct advantages not possible with conventional propagation techniques (Rajasekharan and Ganeshan 2002). Plant tissue culture relies on growing plants on nutrient rich growth substrates devoid of microbes, which results in the production of plantlets without any mutualistic symbiosis (Dolcet-Sanjuan *et al.* 1996). Its more widespread use is restricted by high percentage of lost or damaged plants when transferred to *ex vitro* conditions (greenhouse or field). After transfer from the *in vitro* to the *ex vitro* conditions the plantlets have to correct the above-mentioned abnormalities. In greenhouse, and especially in the field, irradiance is much higher and air humidity much lower than in the vessels. Even if the water potential of the substrate is higher than the water potential of media with sucharose, the plantlets may quickly wilt as water loss of their leaves is not restricted. In addition, water supply can be limiting because of low hydraulic conductivity of roots and root-stem connections. Many plantlets die during this period. Therefore, after *ex vitro* transplantation plantlets usually need some weeks of acclimatization with gradual lowering in air humidity (Pospisilova 1999). The transfer of plantlets cultivated *in vitro* to green house is one of the most important steps in the structural and physiological adaptations during preparation of plantlets. This acclimatization phase is the beginning of autotrophic existence of the plant, with the initiation of physiological processes necessary for survival. During acclimatization, the plantlets must increase absorption of water and minerals as well as the photosynthetic rate (Kapoor 2008).

Although, several techniques have been employed in the past to improve the growth and reduce the mortality rates of plantlets during transplantation, most of them basically aim at controlling environmental conditions, e.g., increasing light intensity and altering the concentration of CO₂ (Kozai and Iwanami 1988) and Laforge *et al.* 1990). It has been reported that biofarming of micropropagated plantlets with arbuscular mycorrhizal fungi (AMF) improves plant performance and plays a significant role in ensuring the health of plantlets (Rai 2001). Moreover, the acclimatization period of micropropagated plantlets can also be shortened by the application of AMF (Salamanca *et al.* 1992). The transfer of plantlets cultivated *in*

vitro to green house is one of the most important steps in the structural and physiological adaptations during preparation of plantlets. This acclimatization phase is the beginning of autotrophic existence of the plant, with the initiation of physiological processes necessary for survival. During acclimatization, the plantlets must increase absorption of water and minerals as well as the photosynthetic rate (Grattapaglia and Machado 1990). Arbuscular mycorrhiza fungi are well known to increase the vigor of plants by increasing absorption of water and mineral nutrients, especially phosphorus. Moreover, AMF can protect host plants from root pathogens and mitigate the effects of extreme variations in temperature, pH and water stress. Successful AMF inoculation at the beginning of acclimatization period has been demonstrated (Branzanti *et al.*, 1992; Estrada-Luna and Davies 2003; Rai 2001). Several examples exist to show the beneficial effects of AMF on improving the growth and development of several *in vitro* micropropagated plant such as coffee, pyrus and potato (Kapoor *et al.* 2008), plum and apple (Fortuna *et al.* 1996), artichoke (Morone Fortunato 2008) and oregano (Morone Fortunato and Avato 2008).

1.6. Mycorrhiza

The most important type of symbiotic plant-fungus associations are mycorrhizas, the statement that “90% of plants are mycorrhiza” has been widely presented in the literature, is not based on scientific data. The actual proportion of angiosperms known to be mycorrhizal is somewhat lower than this, i.e. 82% (Brundrett 2002). Mycorrhizal colonization of roots results in an increase in root surface area for nutrient acquisition. The extrametrical fungal hyphae can extend several cm into the soil and uptake large amounts of nutrients to the host root.

The name mycorrhizas, which literally means fungus-root, was invented by Frank (1885) for non-pathogenic symbiotic associations between roots and fungi. Symbiotic associations are essential for both the partners: fungus which is specialised for life in soils and plants, and root, or other substrate-contacting organ of a living plant, which is primarily responsible for nutrient transfer. Mycorrhizas occur in a specialised plant organ where intimate contact results from synchronised plant-fungus development (Brundrett 2004).

Physically, there are several forms of mycorrhizas, with different forms of hyphal arrangement or associated microscopic structures. At least seven different types of mycorrhizal associations have been recognised, involving different groups of fungi and host plants and distinct morphology patterns. The most common associations are: arbuscular mycorrhiza fungi (AMF), in which Zygomycete fungi produce arbuscules, hyphae, and vesicles within root cortex cells; Ectomycorrhizas (ECM): where Basidiomycetes and other fungi form a mantle around roots and Hartig net between root cells; Orchid mycorrhizas: where fungi produce coils of hyphae within roots (or stems) of orchidaceous plants; Ericoid mycorrhizas: Involving hyphal coils in outer cells of the narrow “hair roots” of plants in the plant order Ericales and the last Ectendo-mycorrhizas: arbutoid and monotropoid associations which are similar to ectomycorrhizal associations, but have specialised anatomical features (Brundrett 2004; Brundrett 1996).

1.7. Arbuscular Mycorrhiza

Arbuscular mycorrhiza are a unique example of symbiosis between two eukaryotes, soil fungi and plants. This association induces important physiological changes in each partner that lead to reciprocal benefits, mainly in nutrient supply (Balestrini e Lanfranco 2006). The AMF are ubiquitous soil microbes constituting an integral component of terrestrial ecosystems forming symbiotic associations with plant root systems of over 80% of all terrestrial plant species, including many horticultural important plants (Kapoor *et al.*, 2008)

Mycorrhiza establishment is known to modify several aspects of plant physiology including mineral nutrient composition, hormonal balance (Harley & Smith, 1983; Azcón-Aguilar & Bago 1994; Barea *et al.* 2002, Taylor and Harrier 2003).

The life cycle of the AM fungus in this widespread symbiosis has a single phase in which the mycobiont is living independent of its host. During that phase, asexually produced chlamydospores are able to germinate in the soil under appropriate water and temperature conditions. (Breuninger and Requena 2004).

After spore germination, establishment of the symbiosis includes hyphal branching, appressorium development after contacting the root, colonization of the

root cortex, formation of intracellular arbuscules, and concomitantly, production of an extraradical mycelium from which spores are eventually formed (Smith and Read 1997).

Usually AM performs best in soils with low or medium fertility because under such conditions it is beneficiary for the host plant to develop symbiosis with the fungi. Due to the unique properties of AM including their very thin and high volume of hypha, which is much finer even than root hairs (this can be very beneficial in a compacted soil), mycorrhizal plants can absorb higher rate of nutrients, resulting in plant-enhanced growth. AM also enhances soil aggregate stability by its network of hypha, which may be very beneficiary in a compacted soil where the soil structure reduces considerably.

AMF are members of the *Glomeromycota* kingdom fungi. Initial studies of the *Glomeromycota* were based on the morphology of soil-borne sporocarps (spore clusters) found in or near colonized plant roots. Distinguishing features such as wall morphologies, size, shape, color, hyphal attachment and reaction to staining compounds allowed a phylogeny to be constructed. The classification based on a consensus of morphological and molecular characters are divide to three families *Glomaceae*, *Acaulosporaceae* and *Gigasporaceae* (INVAM 2009).

Mycorrhiza fungi are relevant members of the rhizosphere mutualistic microsymbiont populations known to carry out many critical ecosystem functions such as improvement of plant establishment, enhancement of plant nutrient uptake, plant protection against cultural and environmental stresses and improvement of soil structure (Smith and Read 1997).

1.8 The objectives

The research described in the following pages was carried out within the PhD activity in Horticulture at Università degli Studi della Tuscia, cycle XXI.

The objective of this thesis was to improve plant quality and speed up the production of micropropagated olive, in order to increase the number of shoots during the proliferation phase and to find the best environmental conditions to induce rooting, and integrated AMF inoculation in an existing micropropagation olive plantlet.

The low percentage of plantlet survival is a problem for expanding the commercial planting of olive plants. The usefulness potential of AMF for agricultural production systems, in particular for the recovery and growth of high-value micropropagated plants, has been shown for several temperate species.

Specifically, this thesis presents data regarding:

- (i) the study of production of inoculum of AMF native from Indonesia,
- (ii) the evaluation of the genetic stability of transgenic olive plant grown *in vitro*,
- (iii) the identification of AMF by morphology and molecular studies,
- (iv) the improvement of micropropagation of olive, and
- (v) the studies of the effect of AMF on micropropagated olive.

II. MATERIALS and METHODS

2.1 Production of arbuscular mycorrhiza

2.1.1 Production of arbuscular mycorrhiza in vitro by using hairy roots of carrot

Unlike saprophytic fungi, the large-scale production of AMF fungi inoculum, due to their obligate symbiotic status, have to produce with host plants associated (Brundrett *et al.* 1996; Dalpé and Monreal 2004; Smith and Read 1997, Douds *et al.* 2004) and are difficult to locate or separated from the root tissue. This experiment was conducted in an attempt to produce AMF by using transgenic roots (hairy roots) with *A. rhizogenes* in vitro culture.

A local cultivar of carrot were purchased in local market and used in these experiments. Carrots were cleaned, washed, peeled and surface sterilised in 5% household bleach (Ace) for 10 min with stirred during sterilization. Further the carrots were washed three times with sterile water and than soaked for 3 min to ethanol solution. Then, carrots were washed several times and rinsed 2 times (5 min each) with sterile distilled water. Each carrot were cut 2 cm from apical and base part, and than was sliced with the disc thickness around 5 mm.

Three isolates of *Agrobacterium rhizogenes* (A4, 9402 and 1855) were used in this experiment. The isolate 1855 and 9402 were grown using media Yeast Manitol Broth (mannitol 10 g/l; K₂HPO₄ 0.5 g/l; Yeast extract 0.4 g/l; MgSO₄·7H₂O 0.2 g/l and NaCl 0.1 g/l), before adding agar the pH were adjusted to 7, and the media solidified with 15 g/l bacto-agar. The isolate A4 was grown using media Manitol Yeast Agar (mannitol 8 g/l; Casamino acids 0.5 g/l; yeast extract 5.0 g/l; amonium sulfate 2.0 g/l and NaCl 4.0 g/l), before adding agar the pH were adjusted to 6.6, and the media solidified with 15 g/l bacto-agar. At 28⁰C. The plates were incubated at 25-28°C in darkness for 36 hours before inoculation on carrot discs.

For inoculation, a loop of 36 hour old bacterial mixture was inoculated on the two side of carrot discs (each loop for each side), and plant were put in to 1% water and media with 10 mg/l AgNO₃ agar media and incubated in dark at 25°C till the callus appeared. The observation of root number and length were recorded every

week for one-month period. The lateral transformed root were transferred to MS media with Carbenicilin (500µg/ml) and than after 1 months were sub culture to the same media, the root were inoculated by leaned and sterilized spore AMF which were washed and sterilized using 400 ppm of streptomycin sulphate.

2.1.2 Production arbuscular mycorrhiza in pots

The inoculums of AMF fungi from Indonesia M1, M2, M3 and M4 (mixed between others) were produced in pots by using *Sorghum bicolor* (L.) Moench var. *sudanese* (Piper) A.S. Hitchc as host plant.

One type of agronomy soil from the field trial (pH 7.940) was used in this propagation method. The medium consisted of soil and sand mixture (1:1), which was autoclaved before its utilization at 121⁰C, for 40 minutes. The bottom holes of pots were sealed with aluminium foil to prevent escape of the medium and poke small holes were made to allow drainage of water.

Seeds of *Shorghum bicolor* (l.) Moench var. *sudanense* (Piper) A.S. Hitchc. were surface disinfected for 10 min using a 10 % of household bleach (Ace): 1 volume of household bleach containing 5% sodium hyphochlorite (NaOCl) plus 9 volumes of water. Then they were washed three times with sterilized distilled water and transferred to a sterile plastic pot containing sterile sand. Pots were watered everyday until germination occurred.

For AMF inoculation and plant growth, five holes were made in media per each pot. Spores of AMF were put into the hole with 3 g inoculums per hole. Spores were covered with soil and the sorghum seedlings were put into the hole. The plants were watered to 12.5% weight of medium and they were grown in the glass house. Once a week, all of the plants were fertilized with a nutrient solution according to Brundtret *et al.* (1996).

Plants were harvested after 16 weeks of growth and after a stressing period for stimulate spores of AMF. At harvest, shoots and roots of sorghum were separated. The roots were used as inoculums with the soil, while the shoots were not used or were discarded.

Spore sieving is important to analyzed number of spores of inocula. Twenty-five g of inoculums of AMF were extracted from the soil samples by wet sieving centrifuged method. The samples is mixed in a substantial volume of tap water and decanted through a series of sieves after following heavy soil particles to settle for few seconds. The inoculums were soaked in 500 ml beaker with water for 30 min were transferred to 50 ml centrifuge tubes, 0,3v of 50% sugar solution was added into the tubes and mixed through and centrifuged at at 2500 rpm for 2 min.

The aquates part was decanted through a series of sieves (420 μm , 250 μm , 100 μm and 45 μm). In case to much debris in inoculums before added glucose solution the soil sample were centrifuged first to separated with the supernatant which contains spore of AMF. The fractions from each screen were colleted into 45 μm soil sieve, and they were washed with running water several time and poured off into a clean petridish. The number of spores for every type of AMF was counted under a dissecting microscope with scale petridish.

2.2 Evaluation of the stability of transgenic genes in olive plants grown in vitro, used for the experiments

Transgenic shoot of the olive cultivar Canino bring the eco15-5 fragment (Canino plantlets were already transformed with *Agrobacterium tumefaciens* strain LBA 4404 containing the *rolABC* gene; Rugini and Caricato 1995) and Canino AT17-2, Canino AT17-3 and Canino AT17-10 (transgenic for the osmotin gene which was delivered through *A. tumefaciens* strain LBA 4404 containing the osmotin gene pKYLX71 under 35S promoter; Rugini and Gutiérrez Pesce 2006) were used for this analysis. The plant material was maintained *in vitro* from several years at 24 ± 1 °C under a 16 h photoperiod (at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, provided by cool white fluorescent lamps).

The DNA of transgenic olive from *in vitro* cultures were extracted based on Doyle and Doyle (1987) protocol. The clean fresh tissue plant was grinded firmly under liquid nitrogen with mortar and pestle, which were pre-cooled. The grinded fine powder plant was put in pre-cooled 2 ml sterile eppendorff tube. One millilitre of extraction buffer, which consisting of 100 mM of Tris/HCl pH 8.0, 20 mM EDTA

pH 8.0, 1,4 M of NaCl, 2% w/v CTAB and 1% of polyvinylpyrrolidone were added immediately and the tube were shaken for 10-15 minutes in room temperature.

Table 2.1: Primer sequence for *rolABC* and *npt II* genes, which were used for evaluating the transgenic gene expression in transgenic olive

Primer	Gene	Sequenze 5' 3'	T _M
<i>rolA (F)</i>	<i>rolA</i>	TCT AAG CTT GTT AGG CGT GCA	55°C
<i>rolA (R)</i>	<i>rolA</i>	CGT ATT AAT CCC GTA GGT CTG	
<i>rol B (F)</i>	<i>rolB</i>	GCG ACA ACG ATT CAA CCA TAT CG	57°C
<i>rol B (R)</i>	<i>rolB</i>	TTT ACT GCA GCA GGC TTC ATG AC	
<i>rol C (F)</i>	<i>rolC</i>	CAG CCC ATC GAC TAA CCA TTA	55°C
<i>rol C (R)</i>	<i>rolC</i>	CCT GTT TCC TAC TTT GTT AAC	
<i>npt II</i>	<i>Osmotin gene</i>	AGAGGCGGCTATGACTGG	55°C
<i>npt II</i>	<i>Osmotin gene</i>	ATCGCCATGGGACGAGAT	

The samples were incubated at 60°C for 30 min, and then were leaved to cool for another 10 min at room temperature. An equal volume (1 ml) of chloroform:isoamyl alcohol 24:1 were added and samples were shaken until an emulsion was obtained. The samples were centrifuged at maximal speed (14000 rpm) for 15 min. The centrifuged made the samples separated in three different layers: the chloroform (organic phase), the cellular debris (interphase) and the diluted genetic material (aqueous phase, which is in the top layer). The aqueous suspension, containing the nucleic acids, was removed by using micropipette into a new sterile 2 ml eppendorf tube. Ice cold isopropanol (0.7 v) was added, and incubated for about 5 min in ice in order to obtain the precipitation of the nucleic acids. A structure fiber of DNA was seen after mixed thoroughly.

The plant DNA was collected by centrifuged for 5 min at the maximal speed (14000 rpm) and the supernatant were discard. The pellet was washed by adding 0.5 ml of ethanol 76% with 0.3 M Sodium acetate and leaving it for 20 min at room temperature, then centrifuged for 1 min at maximum speed (14000 rpm): Then the pellets were washed again using ethanol 76% with 10mM Amonium Acetate for 3 min. To collect the pellet, the samples were centrifuged at max speed (14000 rpm)

for 2 min and the supernatant were carefully discarded. The pellets were dried in the heating block at 37°C in for about 10 min. A volume of 0.2 to 0.4 ml of ddH₂O was added and the DNA was re-suspended in the fridge overnight.

For the RNA purification, 5 µl of RNase (stock solution 10 mg/ml) were added for every 100 µl of DNA solution and incubated for 30 to 60 min at 37°C. Double distilled water was added to reach a volume of 400 µl and then 1 volume of phenol:chloroform:isoamyl alcohol (PCI 25:24:1) were also added: for 400 µl of solution, a 200 µl phenol (saturated with Tris/HCl pH 8) and a 200 µl of chloroform:isoamyl alcohol 24:1. The samples were homogenized by shaking gently until an emulsion appeared, then they were centrifuged for 10 minutes at maximum speed (14000 rpm). The upper aqueous phase were transfer into a new 1.5 ml sterile eppendorf tube and 0.05 volume of 10 M of LiCl and 2.5 volume of ice cold absolute ethanol were added (for 400 µl of solution and 20 µl 10 M of LiCl and 1 ml of Ethanol) and then the DNA were leave to precipitated for at least 20 min at -20°C. Precipitates were collected by centrifuging for 10 min at maximum speed (14000 rpm) and the pellet were washed with 0.5 volume 70% ice-cold ethanol. The washed proceed were repeated for two times. The pellets were dried at 37°C for 10 min in the heating block after centrifuged for 10 min at maximum speed (14000 rpm) and the liquid was discarded.

Pellets were re-suspended gently in an appropriate volume of sterile DNA free water and the quality and concentration of the DNA were checked with spectrophotometer and electrophoresis. The readings were taken at wavelengths of 260 nm and 280 nm using quartz cuvet.

The amplification reaction for osmotin gene expression was performed in a total volume of 25 µl containing of 30 ng template DNA, 2.5 µl PCR suitable buffer, 1.5 µl of 2,5mM MgCl₂, 2.5 µl of 2.5 mM dNTPs, 2.5 µl of nptII-forward, 2.5 µl of primer nptII-reverse, 0.2 µl of *Taq* DNA polymerase, and 13,8 µl of sterile distillate water. Reactions were carried out in a thermal cycler machine, which was programmed as follows: at first at 94°C for 4 min, 35 cycles 94°C for 45 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 7 min (Figure 2.1.)

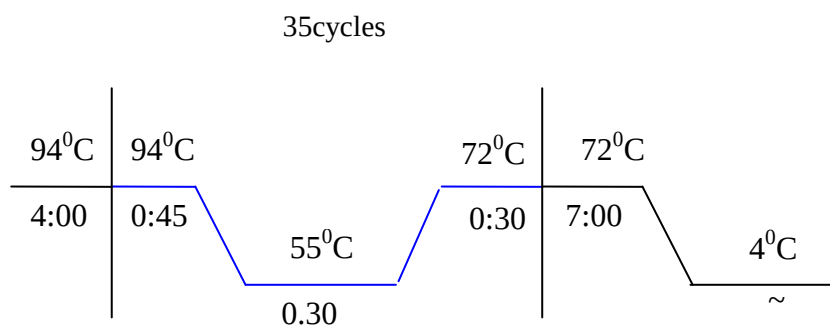


Figure 2.1: Profile DNA amplification for the analysis of gene transgenic expression stability in olive Canino AT17-2, Canino AT17-3 and Canino AT17-10

The reaction of RT-PCR for *rolA*, *rolB* and *rolC* gene expression was performed in a total volume of 25 μ l containing of 1 μ l template DNA, 2.5 μ l PCR suitable buffer 0.75 μ l of 2,5mM $MgCl_2$, 2.5 μ l of 2.5 mM dNTPs, 2.5 μ l of primer-forward, 2.5 μ l of primer -reverse, 0.2 μ l of *Taq* DNA polymerase, and 13. 05 μ l of sterile distillate water (Figure 2.2.)

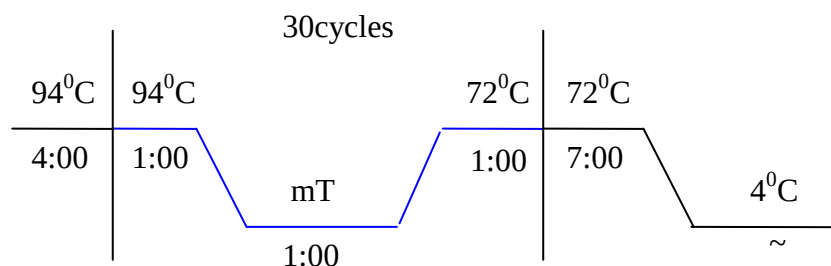


Figure 2.2: Profile DNA amplification for the analysis of the expression of the alien gene *rolA*, *rolB* and *rolC*, as detected in the transgenic Canino plantlets. The value of melting temperature mT was adjusted on the basis of sequencing of primers used, as reported in Table 2.1.

Following amplification, 4 μ l of the PCR reaction mix was combined with 1 μ l of loading buffer and loaded into the wells of the agarose 1.2% (w/v) gel

electrophoresis, which stained with Ethidium Bromide (10 µl/ µl). The Thermal Cycler was programmed as follows at first 94°C for 4 min, 30 cycles at 94°C for 1 min, mTs °C for 1 min, 72°C for 1 min, and 72°C for 7 min. The fragment of DNA were seen and photographed under UV light.

2.3 Identification of arbuscular mycorrhiza

2.3.1 Morphological identification of arbuscular mycorrhiza

The arbuscular mycorrhizal fungi were taken from an agricultural land at Lombok (8° 33' 54" S, 116° 21' 3.6" E), an island in West Nusa Tenggara province, Indonesia. The agricultural lands were dominated by tobacco. The soil samples were stored in polyethylene bags at 4 °C until processed. Soil samples, used for this identification were extracted from pot cultures, utilized for AM fungal reproduction. Single fungal productions using *Sorghum bicolor* (L.) Moench as host plant were produced for 4 months in a 1:1 (by volume) soil and sand in plastic pot. Three types of AMF natives from Indonesia, were named M1, M2 and M3.

Spores were collected from soil samples and were extracted by using the wet sieving method. Samples were mixed in a substantial volume of tap water and decanted through a series of sieves. Washings were repeated until the water was clear. The sieves size used were 500 µm, 100 µm and 50 µm. The fractions from each screen were put in Petri dishes. The presence of AMF spores were checked under a stereomicroscope and collected individually one by one, using micro forceps (Martin, Germany) or micropipettes (200µl), according to their morphology and colour. The spores were collected until the necessary amount for identification.

Diagnostic slides were made for AMF identification. A hundred spores were collected for diameter size measurement; and 20 spores were mounted in lactic acid reagent on each slide. Spore diameter size was measured with ocular micrometer. 25 spores were put in polyvinyl alcohol-lactic acid-glycerol (PVLG) and Meltzer's reagent to see spore wall layers and Meltzer's staining reaction. Spores were examined to record their morpho-taxonomic features and their identification was based on (Schenck and Perez, 1987) and INVAM (2009).

Roots were washed in tap water and cleared in 10% KOH boiling in this solution for 30 minutes. After, roots were washed with the tap water for several times and were left in 2 % HCl for 10 minutes. At last roots were stained in trypan blue solution (0.05% trypan blue in 90% lactic acid), heated for 15 minutes, and after moved to lactic acid solution

2.3.2 Molecular identification of arbuscular mycorrhiza

Two different types of AMF identified as *Gigaspora albida* and *Gigaspora rosae* by morphological methods, were molecularly characterised by ITS (Internal Transcribed Spacer) region and partial SSU (Small SubUnit) ribosomal DNA (rDNA) sequences.

DNA was extracted from single spore of the isolates M1 and M3. Clean, good, fresh and healthy spores from two type of *Gigaspora* where taken from the same cleaning procedures for morphological identification and collected one by one in eppendorf tubes with clean distilled water (CDW) under a dissecting microscope. Spores were sonicated two times (1 min each, RF frequency 42 KHz +/- 6 %) in a B-1210 cleaner (Branson Ultrasonics, Soest, The Netherlands) and washed two times with CDW representatively and three times in sterile distilled water (SDW) on laminar air flow cabin. Cleaned spores were collected in sterile eppendorf tubes and surface disinfected with 2% (w/v) chloramine-T (siqma) and 400 ppm of streptomycin sulfate (sigma) for 20 minutes. Then were rinsed four times in SDW. Clean and sterile spores were selected again and transferred in sterile 1.5 ml eppendorf tubes before DNA extraction. Five single spores were analysed for each morphotype of *Gigaspora*.

According to the protocol described by Redecker *et al.* (1997) and Turrini *et al* (2008) a single spore was crushed using a sterile glass pestle (hand made) in 2 µl of 0.25 M NaOH (in a total volume 10µl of 0.25 M NaOH: a volume 2 µl solution were put first, in order to prevented the spore running away during the crushed process) the other 8 µl of 0.25 M NaOH were added before incubation in boiling water (1 min). Five µl of 0.5 M Tris-HCl (pH 8) and 10 µl of 0.25 M HCl were added and the microtubes were dipped again in boiling water (2 min). Each DNA extracts was diluted 10 times and 1 µl was directly used for PCR amplification.

PCR conditions of SSU sequences

DNA extracts from single spores of *Gigaspora* morphotypes were used to analyse partial SSU sequences. A volume of 1 µl from a 1:10 dilution of template DNA extracts were amplified in total 25 µl of PCR reaction mix using 0.625 U of AmpliTaq Gold DNA Polymerase (Applied Biosystem, Milan, Italy). The universal eukaryotic primer NS31 (10 pmol) as a forward primer (Simon *et al.* 1992) and the primer AM1 (10 pmol) as reverse primer (Helgason *et al.* 1998) were used to amplify a small portion (550 bp) of arbuscular mycorrhizal fungal SSU rDNA sequences (Douhan *et al.*, 2005 and Turrini *et al.*, 2008) together with 0.2 mM (each) dNTPs, 1.5 mM MgCl₂ and the manufacturer's reaction buffer. The amplifications were ran in the thermal cycler apparatus (Eppendorf Mastercycler – Pisa University)

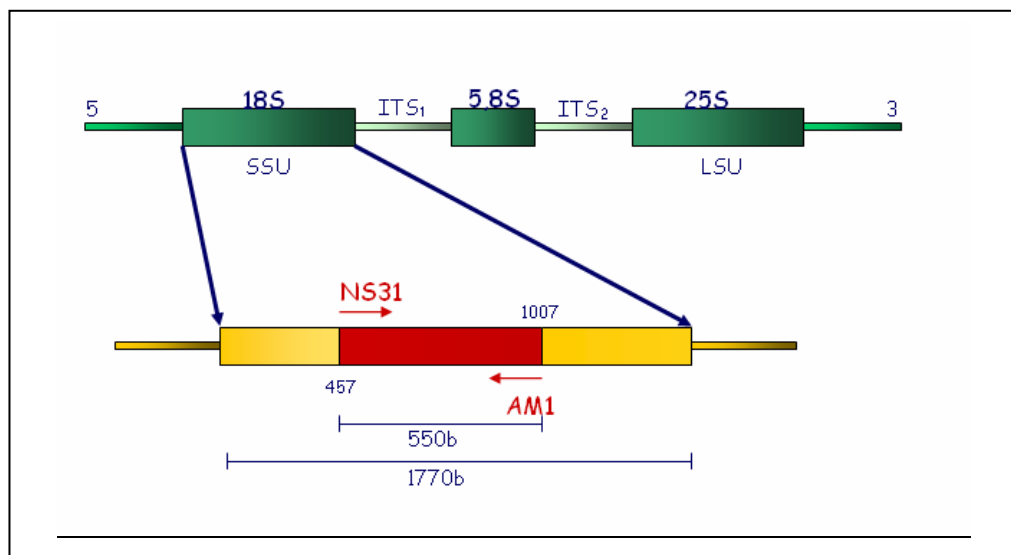


Figure 2.4. Scheme of the 18S gene and the region between primers NS31 and AM1 (Helgason, 1998).

The Thermal Cycler was programmed as follows: 95°C for 9 min, 10 cycles at 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, 24 cycles at 95°C for 30 s, 58°C for 1 min, 72°C for 2 min, 1 cycle at 95°C for 30 s, 58°C for 1 min, and 72°C for 10 min.

PCR conditions of internal transcribed spacer regions (ITS)

The same DNA extracts which were utilized for SSU sequences analyses (a volume of 1 µl from a 1:10 dilution of template DNA) were used for analysing the internal transcribed spacer regions between the primers ITS1F (5'-CTTGGT CAT TTA GAG GAA GTA A -3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC -3') (Gardes and Bruns 1993; Turrini *et al.* 2008). The internal transcribed spacer region between ITS1F/ITS4 regions were amplified in a total volume of 25 µl PCR reaction mix.

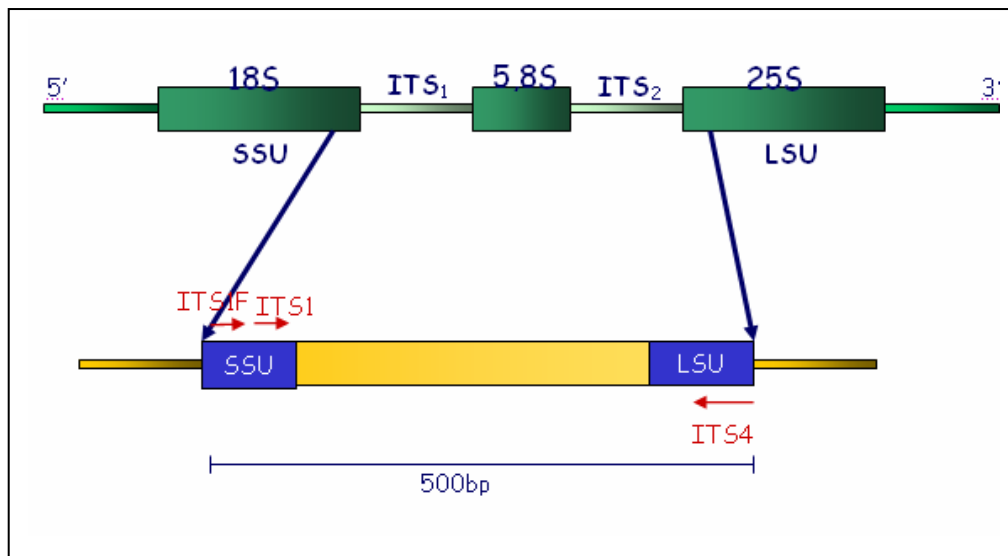


Figure 2.5. Scheme of ITS region between primers ITS1F and ITS4

Final concentration of PCR mix components were 0.2 mM (each) dNTPs, 1,5 mM of MgCl₂, 10 pmol of each primer, 0.625 U of AmpliTaq Gold DNA Polymerase (Applied Biosystem) and the manufacturer's reaction buffer. Reactions were carried out in a thermal cycler machine with amplification program consisted of

an initial denaturation for 5 min at 95°C, followed by 4 cycles at 95°C for 30 s, 54°C for 30 s, 72°C for 1 min, 34 cycles at 95°C for 30 s, 53°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 10 min.

The seminested PCR reactions (Renker *et al.*, 2003) were performed by diluting (1:100) the first PCR amplicons and using 2 µl of dilutions as template for the second reaction in a final volume of 50 µl, according to the protocol described by Turrini *et al.* (2008), by using the primers ITS1 (5'-TCC GCA GGT TCA CCT ACG GA-3') and ITS4.

PCR mix components were 0.2 mM dNTPs, 2.5 mM MgCl₂, 10 pmol of each primer, 0.625 U of AmpliTaq Gold DNA Polymerase and the manufacturer's reaction buffer. Reactions were carried out following a touch down protocol to reduce the formation of spurious by-products during the amplification process. PCR reaction mix was preheated at 95°C for 5 min, then the annealing temperature was set 14°C above the expected annealing temperature (55°C) and lowered with 2°C every third cycle until 55°C, at which temperature 14 additional cycles were carried out. Cycles were performed as follows: 95°C for 30 s, annealing temperature 30 s, 72°C for 1 min. The end of the last cycle was followed by an extension step at 72°C for 10 min.

Cloning and sequencing

Three PCR products from SSU region and three semi-nested PCR products from ITS regions were purified by using Montage PCR Centrifugal Filter Devices Kit (Millipore) with a final elution volume of 20 µl. Purified products were cloned using Qiagen PCR Cloning Kit according to the manufacture's instructions (Qiagen, Milan, Italy). Putative positive clones containing recombinant plasmid were purified by the Gen Elute Plasmid Miniprep Kit (Sigma, Milan Italy) and screened using standard SP6/T7 amplifications, followed by a PCR using NS31/AM1 (for SSU) or using ITS1/ITS4 (for ITS), as described above. NS31/AM1 amplification products were sequenced forward and reverse at BMR Genomics s.r.l. (University of Padua, Italy as connection from the Department of Agricultural Plant Biology, University of Pisa), and ITS1/ITS4 amplification products were sequenced forward and reverse at

ABI Prism 310 genetic Analyzer machine (Dipartimento di Agrobiologia ed Agrochimica, Università degli studi della Tuscia-Viterbo).

SSU and ITS sequences, which were obtained from the two morphotypes were aligned using both the Multalin program and the ClustalW program (Chenna *et al.* 2003) with sequences of *Gigaspora* available in GenBank. After alignment, a phylogram was generated by TREECON for Windows by using the Kimura 2 parameter (van de Peer and de Wachter 1994). The confidence of branching was assessed using 1000 bootstrap resamplings.

Restriction Analysis of PCR-Amplified ITS rDNA

The amplified ITS rDNA were digested with the restriction enzymes *Mbo*I as described by Redecker *et al.*, (1997). Each digestion was performed separately in a sterile 1.5 ml eppendorf tube containing 1 µl of restriction enzyme *Mbo*I, 2 µl of the manufacturer's buffer and 4 µl of the amplified rDNA, and H₂O to a final volume of 20 µl. The reactions were incubated over night at 37°C. Digested fragments were separated on a 2% Metaphore gel and stained with ethidium bromide. The size of the restriction analysis were measured by using a DNA marker.

2.4 Micropropagation of olive

In attempt to improve the quality and speed up the production of micropropagated olive *in vitro*, some experiments have been carried out in order to increase the number of shoots during proliferation phase and to find the best environmental conditions to induce rooting.

2.4.1 Effect dikegulac in micropropagation of olive

To increase number of shoots of olive *in vitro* by reducing apical dominance, the effect of dikegulac in the following cultivar: Canino, Frantoio, Moraiolo, Rosciola and Piantone di Moiano have been studied. All explants were collected from *in vitro* shoots established some years earlier. The explants were cultured on Rugini Olive Medium (ROM) (Table 2.2.) with 0.45 µM zeatin and 3.6% mannitol,

the pH was adjusted to 5.8 before solidified by adding plant agar (0.6% w/v) and then media were sterilized by autoclaved for 20 min at 121⁰C.

Two nodal shoots were excised from 40-day-old *in vitro* culture and placed into new jars with the same medium of multiplication plus a range of dikegulac (Sigma) with concentrations (0.0, 16.9, 33.8, 66.7, 100.5 and 133.4 μ M) and dikegulac were filter-sterilized and added to the medium after autoclaving. All cultures were kept in a growth chamber at 24 $\pm$ 2⁰C under a 16 h photoperiod (at 40 μ lmol m⁻² s⁻¹ light intensity, provided by cool white fluorescent lamps). Shoot proliferation was evaluated after 40 days of culture and number, length of shoots and plant survival were recorded.

2.4.2 Effect of three different light conditions in the development of roots for the cv Canino WT and transgenic *rolABC* Canino

In attempt to improve rooting two type of olive plant *in vitro*, three types light culture condition have been studied. Olive *in vitro* cv Canino wt and Canino *rolABC* were rooted in two to three nodes stage in rooting medium consisted of ROM supplemented with concentrations of IBA (0.4mg/l) and putrescine (160 mg/l). Olive shoots were incubated in the dark at 24 $\pm$ 2 ⁰C for 4days and then were transferred to 24 $\pm$ 2⁰C under a 16 h photoperiod (at 40 μ lmol m⁻² s⁻¹ light intensity, provided by cool white fluorescent lamps) the explant were put in three different light condition: L1 put in dark for four days and transferred to the light in growth chamber; L2; plants were always in completely darkness from the beginning of rooting phase, and L3: were always in the light during all rooting process. The number of roots and length were recorded in the end of experiment.

2.4.3 Multiplication and rooting in vitro.

The olive cultivars used in this experiment were: Canino, Moraiolo, and transgenic Canino eco15-5, Canino AT 17-2, Canino AT 17-3 and Canino AT 17-10 for screening AMF. All explants were collected from *in vitro* shoots established some years earlier. The explants were cultured on Rugini Olive Medium (ROM) with 0.45 μ M zeatin and 3.6% mannitol, the pH was adjusted to 5.8 before solidified by

adding plant agar (0.6% w/v) and then media were sterilized by autoclaved for 20 min at 121⁰C

Uninodal shoots were excised from 40-day-old *in vitro* culture plantlets and used for multiplication. After one culture period, the new shoots were transferred to new and the same multiplication media. Plants were continuously cultured until the number of plantlet reached for the experiment. All cultures were kept in a growth chamber at 24 ± 2⁰C under a 16 h photoperiod (at 40 µmol m⁻² s⁻¹ light intensity, provided by cool white fluorescent lamps)

Olive shoots were rooted *in vitro* at the two to three nodes stage in rooting medium consisted of ROM supplemented with concentrations of IBA (0.4mg/l) and Putrescine (160 mg/l). Olive shoots were incubated in the dark at 24±2 °C for 4-5 days and then were transferred to 24 ± 2⁰C under a 16 h photoperiod (at 40 µmol m⁻² s⁻¹ light intensity, provided by cool white fluorescent lamps) for 3 weeks.

Table. 2.2: Composition of basal Rugini Olive medium used for the *in vitro* culture of olive plants.

Micro Elements		
CoCl ₂ .6H ₂ O	0.025 mg/l	0.11 µM
CuSO ₂ .5H ₂ O	0.25	1.00 µM
FeNaEDTA	36.70	0.10 mM
H ₃ BO ₃	12.40	0.20 mM
KI	0.83	5.00 µM
MnSO ₄ .H ₂ O	16.90	0.10 mM
Na ₂ MoO ₄ .2H ₂ O	0.25	1.03 µM
ZnSO ₄ .7H ₂ O	14.30	49.75 µM
Macro Elements		
CaCl ₂	332.16 mg/l	2.99 mM
Ca(NO ₃) ₂	416.92	2.54 mM
KCl	500.00	6.71 mM
KH ₂ PO ₄	340.00	2.50 mM
KNO ₃	1100.00	10.88 mM
MgSO ₄	732.60	6.09 mM
NH ₄ NO ₃	412.00	5.15 mM
Vitamins		
Biotin	0.05 mg/l	0.20 µM
Folic acid	0.50	1.13 µM
Glycine	2.00	26.64 µM
Myo-Inositol	100.00	0.55 mM
Nicotinic acid	5.00	40.62 mM
Pyridoxine HCl	0.50	2.43 µM
Thiamine HCl	0.50	1.48 µM

2.5 Inoculation of arbuscular mycorrhiza in micropropagated olive plants

The plantlets were inoculated at time of transferring from *in vitro* to *ex vitro* environment with four different arbuscular mycorrhiza fungi M1, M2, M3 and M4 which described in Table 2.3

Table 2.3: The list of arbuscular mycorrhiza fungi used in the experiment

Arbuscular mycorrhiza	code
<i>Gigaspora rosea</i> *	M1
<i>Glomus intaradices</i> *	M2
<i>Gigaspora albida</i> *	M3
Mixed spora inoculum	M4

* The arbuscular mycorrhiza name were known after the morphological identification

Arbuscular mycorrhiza M1, M2, M3 and M4, and M0 (no inoculated) were applied to the cultivars Moraiolo, Canino, Canino AT17-2, Canino AT17-2, Canino AT17-2, Canino *rolABC*).

Rooted plants were transplanted into sterile pot containing sterile media 50:50 (v/v) mixture of soil and sand when they presented two to four nodes. The inoculum of AMF, containing spores, infected root and hype, was placed adjacent to and below the roots in the substrates at any plant during transplanting.

The pots where maintained in a box for 21 days with transparent polyethylene film, which was progressively removed to reduced the relative humidity inside, plants were further maintained under natural photoperiod conditions in glasshouse. Controls consisted of the remaining non-inoculated plantlets. During nursering plants were watered and fertilized with 10 ml of half Hoagland's solution/week for 6 months and 20 ml of full doses until the end of the experiment. Each treatment comprised 10 replicates, consisting of one plant per pot. The survival rate of acclimatization was calculated after one month of transferred to *ex vitro* environment. At the end of the experiment fresh weights (g), root length (cm), plant length (cm), shoot volume (mm³) and root volume were recorded (mm³). The plants

were firmly separated from substrate, rinsed with running water and dried with tissue paper. Fresh weights, shoot and root length as well as volume were recorded. Estimation of root colonization were carried out by washed and clean the root systems to remove soil particles and partially digested in 10% KOH (w/v) at 90°C for 30 min. After digestion, the roots were stained with 0.05% trypan blue in lactophenol (v/v) at 90°C for 15 min. Fungal structures (hyphae, vesicles, arbuscules) were stained in blue. Mycorrhizal colonization was estimated using the grid-line intersect method, described by Brundtrett *et al.* (1996).

Plant leaf area was measured by using UTHSCSA Image Tool version 3, an image processing and analysis program provided by the University of Texas. Leaves were taken randomly from the plants starting up to the fourth node for each treatment. The quantification of chlorophyll has been done on fresh leaf discs of 0.5 cm diameter, with a variable number for each sample until a weight of 0.1 g was reached. The leaves tissue were soaked in 10 ml of DMF (N,N-Dimethylformamide) All surface of tubes were closed with aluminium foil to prevent the light come inside. The leaves were incubated at 4 ° C for 48 hours.

The supernatant were read with spectrophotometer calibrated at $\lambda = 703$ nm with DMF. The readings of the samples were made at $\lambda = 664$ nm and $\lambda = 647$ nm; following the protocol described by Moran (1982). The value of absorbance was translated into the amount of chlorophyll in accordance with the formulas below :

Chlorophyll A = 12.64 (Absorbance 664) - 2.99 (Absorbance 647)

Chlorophyll B = 23.26 (Absorbance 647) - 5.6 (Absorbance 664)

Total chlorophyll = 7.04 (Absorbance 664) + 20.27(Absorbance 647)

Statistics

The experiments were set up according to factorial designs. The results were analysed The R Project for Statistical Computing a free software environment for statistical computing and graphics, version 2.81 (R, 2009). For all characteristics studied the statistical significance of differences between means were determined using the least significant difference (LSD).

III. RESULTS and DISCUSSION

3.1 Production of arbuscular mycorrhiza

3.1.1 Production of arbuscular mycorrhiza in vitro by using hairy roots of carrot

Carrot discs without *A. rhizogenes*, placed in water agar medium, after one month, showed neither root formation nor callus. On the other hand carrot discs treated with AgNO₃ produced roots in a small number and short length only after 21 days in culture.

In carrot discs, treated with *A. rhizogenes*, the roots started to appear after 7 days; the first ones that appeared looked very delicate, short and thin. In both media (water agar and AgNO₃) the roots initiated from both sides of the discs in high number and elongated quickly (Figure 3.1).

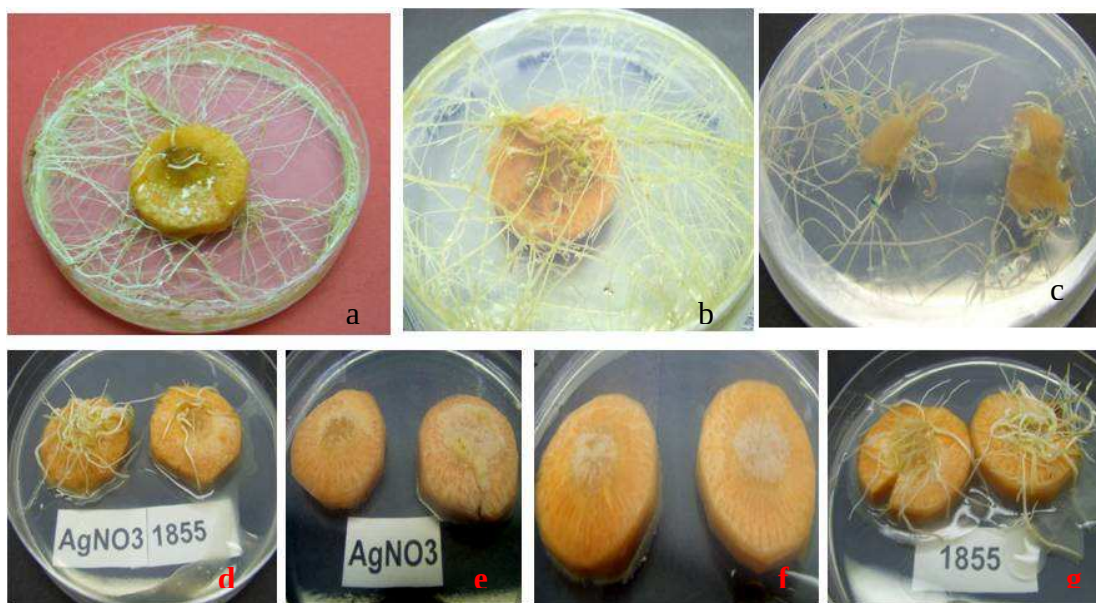


Figure 3.1: Lateral branches of transformed roots (a, b, c). Initiation and growth of transformed roots on carrot discs inoculated with *A. rhizogenes* 1855 in silver nitrate medium (d) and in water agar medium (g). Control showed callus formation without root in silver nitrate medium (e) and in water agar medium (f).

As shown in the Figure 3.2, the highest number of root after 4 week of culture and inoculation appeared in 1% WA and strain 1855 in carrot discs. The carrot discs put in 1%WA with the strain A4 showed the best length of the roots (3.68 cm) followed by AgNO₃ strain A4. Carrot disc which were put in media with AgNO₃ showed the lowest number and root length; the addition of AgNO₃ more likely prevent long root transformation.

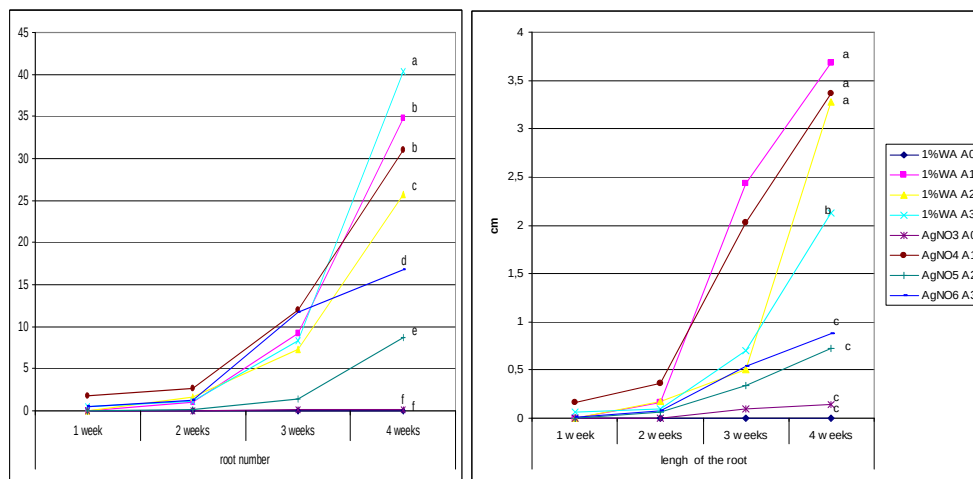


Figure 3.2: Number and length of carrot roots in media 1%WA (water agar) and AgNO₃ from 1-4 weeks. The letters showed the significant different in level $P > 0.05$ at 4 week (w = week; A0 = non inoculants, A1 = *A. rhizogenes* strain A4, A2 = *A. rhizogenes* strain 9402, A3 = strain *A. rhizogenes* 1885)

Although carrot is one of the most amenable species of plant for hairy root production (Danesh *et al.* 2006; Dhankulkar *et al.* 2005), there were some problems during our studied to optimizing the production of transformed hairy root, the subcultures failed for several times caused by the hairy root decay, the quality of roots and contamination. Due to those problems and the limited time that we had, the production using pot culture technique seems a good decision.

3.1.2 Production arbuscular mycorrhiza in pots

Since AMF are obligated symbionts, they were produced in pots by using sorghum as host plants (Gerdeman and Trappe 1974; Smith and Read 1997; Bedini *et*

al. 2007). After 4 months, different density of spores between the different types of AMF were observed:

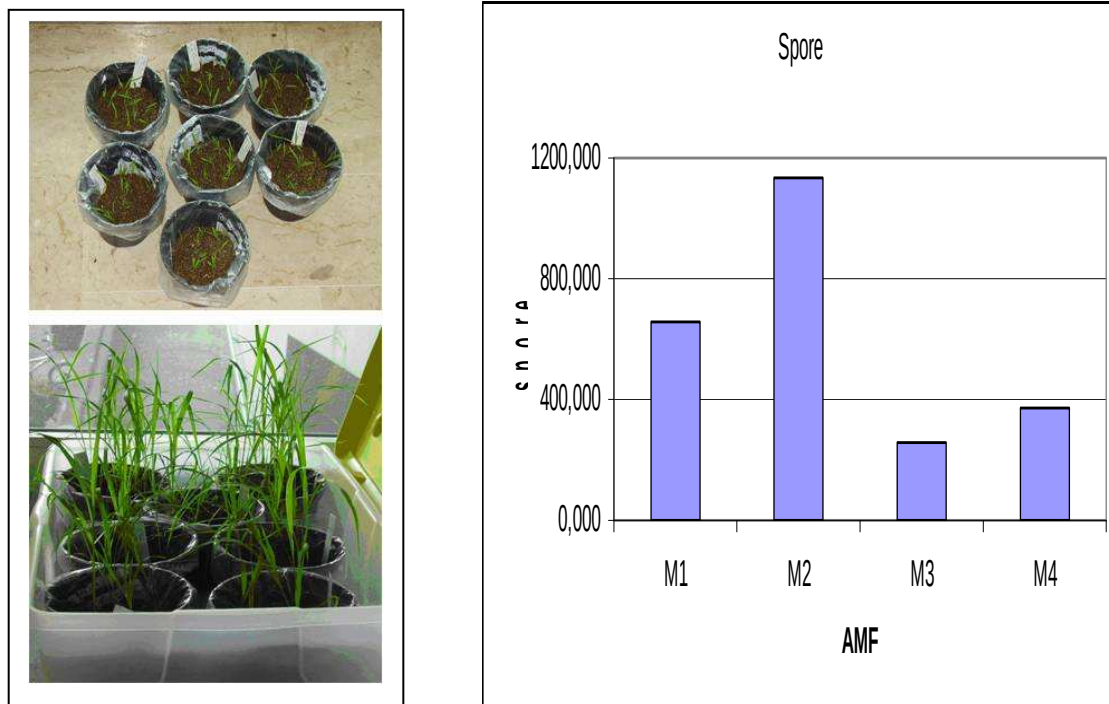


Figure 3.3: Arbuscular mycorrhiza production. Spores density among the different types of arbuscular mycorrhiza native from Indonesia

This AMF activity is very important due to the needs of inocula for screening the their activity in micropropagated olive plants. The inocula consisted of infested soil with spores, external mycelium and infected root fragments

3.2 Evaluation of the stability of transgenic genes in olive plants grown in vitro

Plant DNA from *in vitro* olive plants of Canino (control and transgenic) was extracted with the aim to verify the stable insertion of *rolABC* gene from the Ri DNA of *Agrobacterium rhizogenes* and the *osmotin-nptII* gene. PCR reactions were carried out by using specific primers for each gene like were described at material and method. After several years of *in vitro* cultures, the molecular analysis were been repeated on tissue samples of olive plant *in vitro* to analyzed the transgenic gene

stability. The olive plant DNA of Canino and transgenic Canino AT17-2, Canino AT17-3, and Canino AT17-10 were analyzed by PCR to confirm the presence of *nptII* genes.

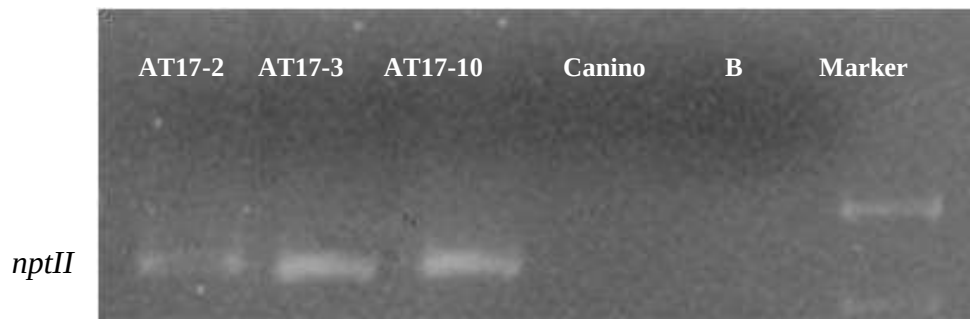


Figure 3.4: Electrophoresis profile of the amplification products of transgenic Canino AT17-2, AT17-3, AT17-10, and Canino WT with pair reverse and forward *nptII* primer. The transgenic cultivar of olive showed a positive fragment.

The PCR analysis showed that the fragment of the *nptII* primers, was present in transgenic Canino clone: AT17-2, AT17-3 and AT17-10, but not in the Canino WT (Figure 3.4.). Similar results were obtained for the transgenic plant of Canino Eco15-5 for *rolABC*, in which it was found the stable integration of the *rol* genes in shoots and stem of fresh plants after several years of *in vitro* culture

In Figure 3.5, it showed that the product of amplification in the tissues of leaves and stems of transgenic plants Canino Eco15-5. The products of amplification of cDNA extracted from plant tissues of wild type cultivar product is visible which indicated there are no amplification product with the pair primers *rolA*, *rolB* and *rolC*.

A complete analysis of transgenic plants also involves the evaluation of the transgenic expression using standard molecular techniques such as PCR (Rugini *et al.* 1999; Rugini and Gutiérrez-Pesce 2006; Flachowsky *et al.* 2008). This is important due to major problems in most transformation systems, such as regeneration of escapes and chimeric shoots that emerge during transgenesis (Caboni *et al.* 2000; Rugini *et al.* 1999)

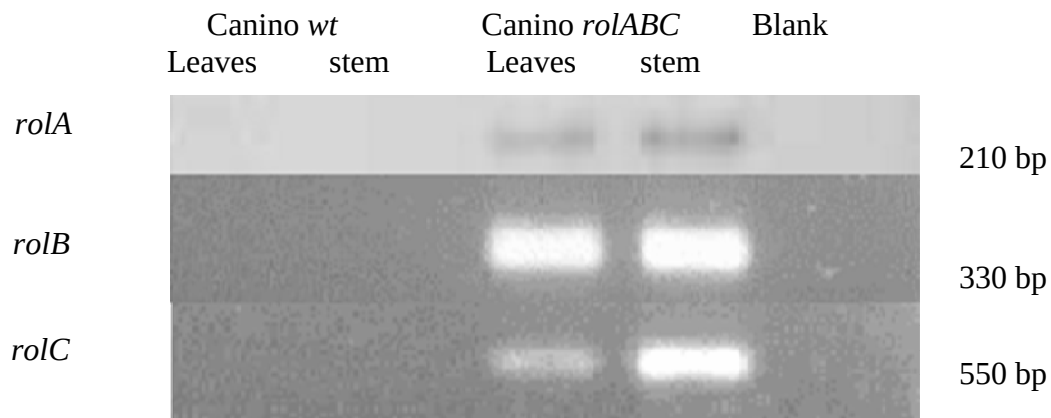


Figure 3.5: Electrophoresis gel of DNA amplifications products of transgenic plants Eco 15-5 and WT of Canino using specific primers for *rolA*, *rolB* and *rolC*. Lane 1-2: Canino WT; lane 3-4: transgenic Canino; lane 5: blank.

Although different studies on transgenic trees have described that the instability of the insertion of alien genes, during the *in vitro* subculture (Flachowsky *et al.* 2008), in our cases the detection of the *rol* genes after subcultures clearly indicate that the insertion is stable. In fact the PCR tests repeated many times did not show dedifferentiation during all the period of cultivation *in vitro*.

3.3 Identification arbuscular mycorrhiza

3.3.1 Morphological identification of arbuscular mycorrhiza

Three types of AMF native from Indonesia, named M1, M2 and M3, were identified by their morphological spore characterization mainly based on the mean of spore diameter distribution, spore colour, wall structure and hypha attachment (INVAM 2009; Schenk and Smith 1982; Schenk and Perez 1987; Nicolson and Schenk 1979; Bentivenga and Morton 1995). The observations were done to a hundred of spores of each of the three types of AMF.

The morphologic characters of the spores were recorded one by one. The M1 spores had pale creamy pink colour with round and globosely shape. Under light pressure in the microscope and staining with Melzer reagent solution it was possible to see the complete wall structure of 2-4 inseparable layers wall (Figure 3.6).

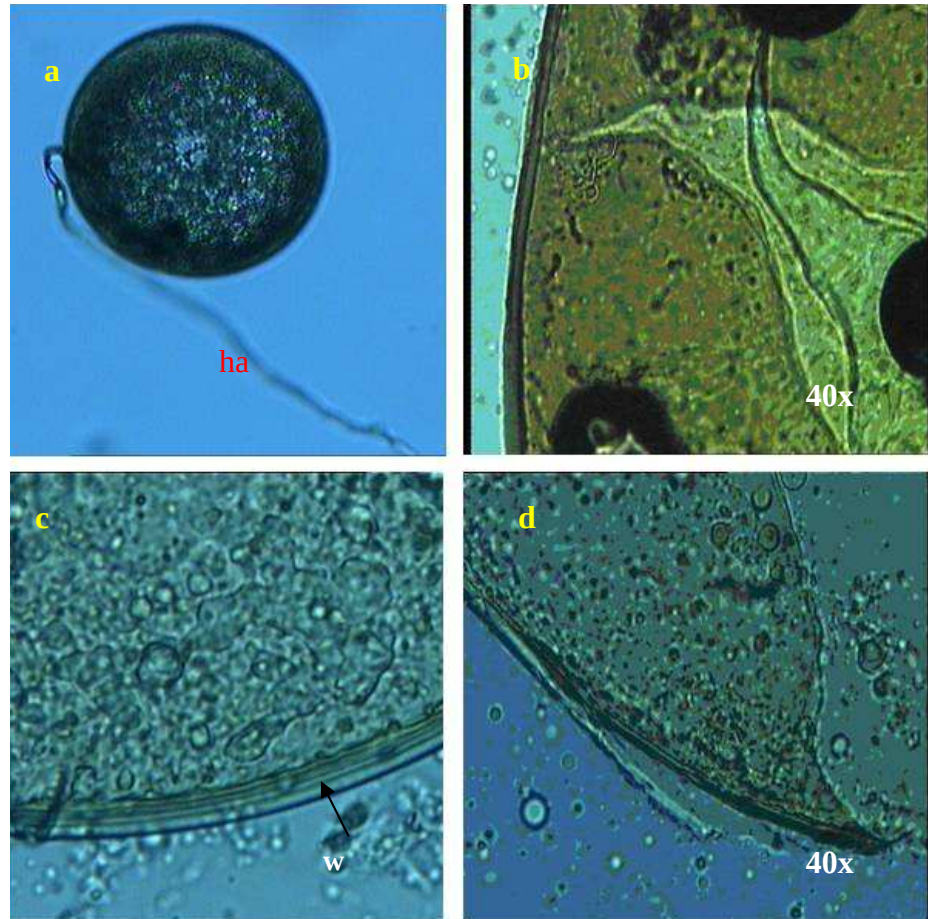


Figure 3.6: Light microscope of *Gigaspora rosea* a: Single spore with hyphal attachment (ha); b: spore wall (w) layer in PVLG reagent; c; d: crushed spore in Melzer's staining layer showed spore wall group structure.

As shown in Figure 3.7, the spore diameter size distribution ranged from 180 to 270 μm with mean 227.2 μm . This size was included into the distribution diameter class mentioned by Nicolson and Schenck (1979) and Schenck and Perez (1987) for the group of *Gigaspora* and mainly for the species of *G. rosea*. The width of subtending hyphae was between 22.5-45 μm (mean 33.4 μm), and this value is included in to the range size of the species of *Gigaspora roseae*, which was reported

by Schenck and Perez (1987), 24-36-50 μm , and by INVAM (2009), 32-45 μm . After the identification, M1 samples will be referred as *Gigaspora rosea* herein after.

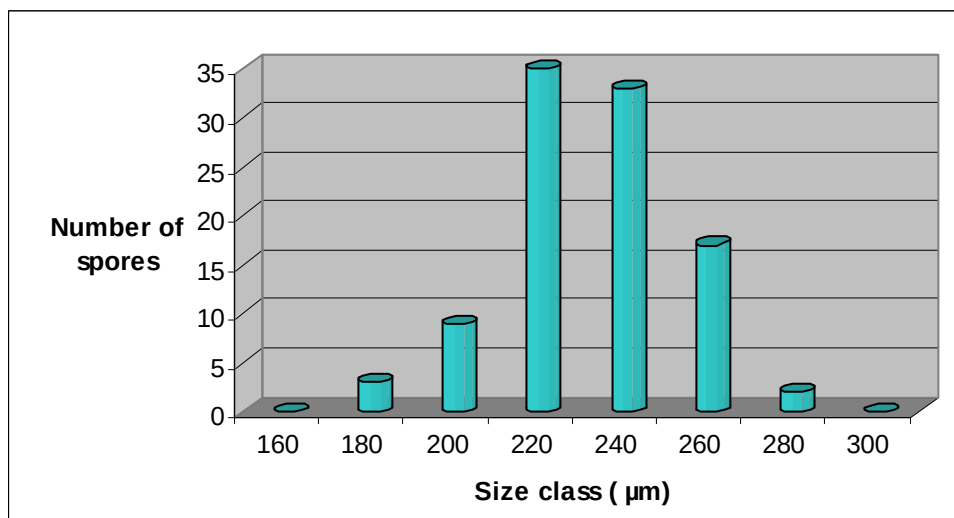


Figure 3.7: Distribution size diameter (μm) of *Gigaspora rosea* spores

From the M3 spores observation we found that the shape of these spores were round and globosely, with cream pale light green colour, with spore size diameter distribution between 170 to 280 μm , and mean 232,5 μm (Figure 3.8). This value was included into to range size of *Gigaspora albida*, mentioned by Schenck and Smith (1982) and Schenck and Perez (1987).

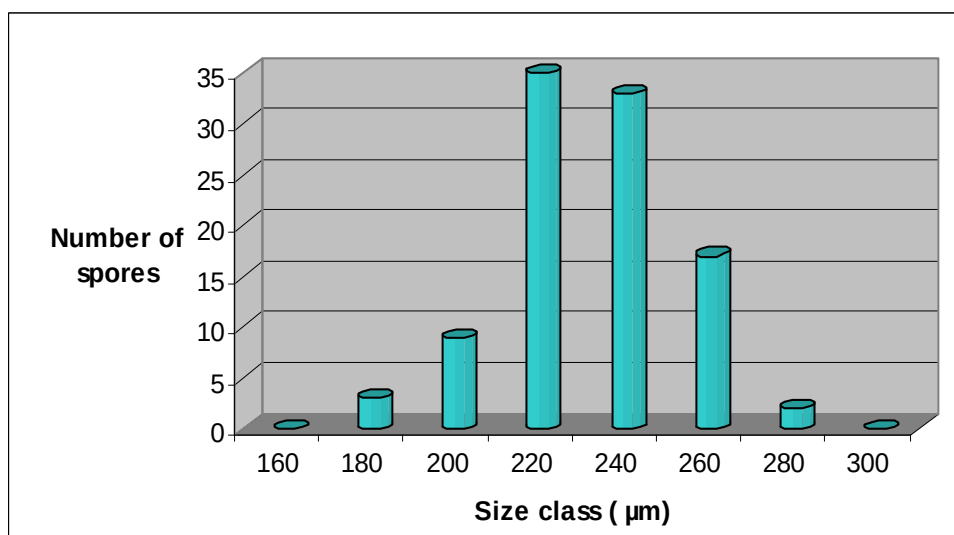


Figure 3.8: Distribution size diameter (μm) of the spores of *Gigaspora albida*.

The M3 width of subtending hypha was between 23-48 μm (mean 30,69 μm), which was included in to the distribution class of *Gigaspora albida* as described by Schenck and Perez (1987), and INVAM (2009). The observation of the M3 spores wall showed three layers: an outer, an inner and a germinal layer. After the reaction with Melzer's reagent solutions the outer laminar layer did not showed strong enough colour compared with the inner and germinal layer, which turned to dark purple (Figure 3.9). The outer wall was thinner and stickier than inner layer. Under light pressure is possible to see 2-3 layers of wall which inseparable (Figure 3.9). After the morphological characterization the arbuscular mycorrhiza M3 will be referred to as *Gigaspora albida* hereinafter.

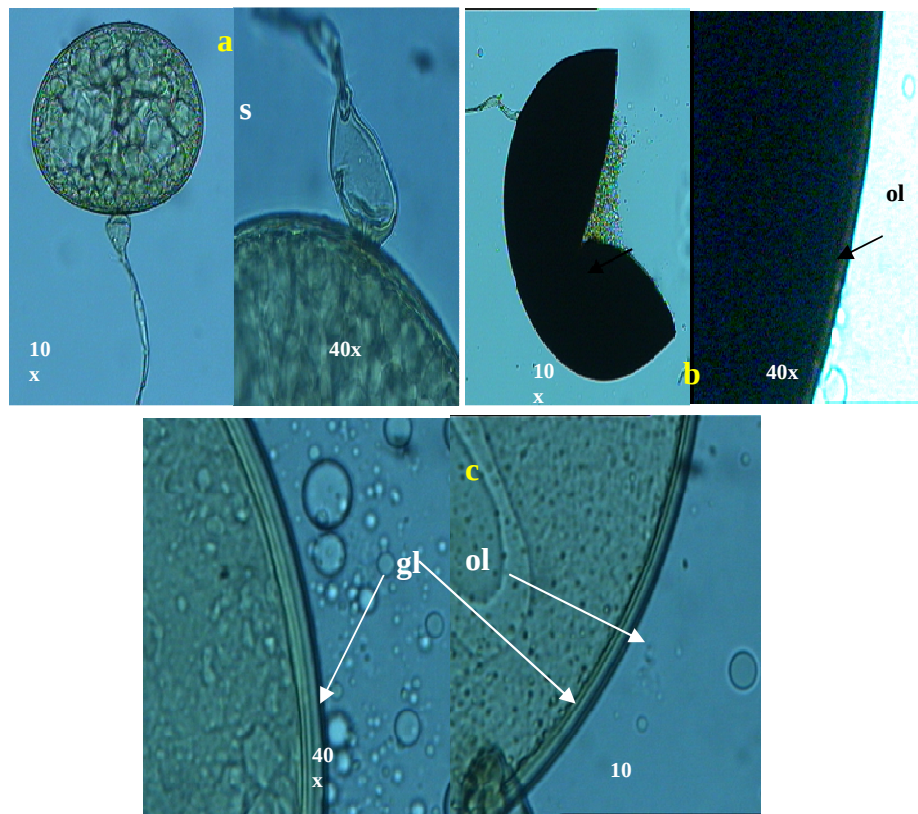


Figure 3.9: Light microscope of *Gigaspora albida*. a: Single spores with bulbous sporogenous cell (s). b: crushed spore in Melzer's staining reaction, ol: outer layer is lighter than inner layer and germinal layer. c: wall layer in PVLG. ol: outer layer, il: inner layer, gl: germinal layer.

The spores diameter size distribution of M2 samples (Figure 3.10) ranged between 90-180 μm , and this value is included in to the range size of the species of *Glomus intraradices* (INVAM), From the M2 spores observation we found with white pale cream colour and round globosely shape.

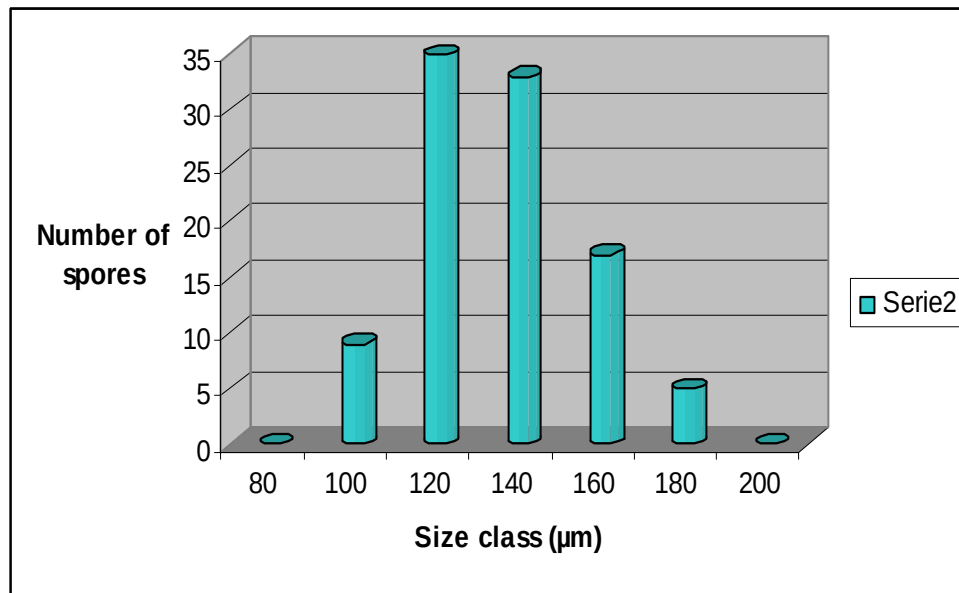


Figure 3.10: Distribution size diameter (μm) of *Glomus intraradices* spores

As showed in Figure 3.11, the spores exhibited in three layers, with the outer layer less colour in Melzer's reagent than others layers. Moreover spore wall looked to have a fragile structure. The outermost layer of *Glomus intraradices* was always degraded and decomposed naturally from the action of microorganisms, and for this reason it appears granular and may accumulate some debris (INVAM 2009). The inner layer in Melzer's reagent showed darkness colour than the outer wall. This layer forms simultaneously in the wall of the subtending hypha. Root formation showed the vesicle, arbuscular form and spores. AMF code M2 hereinafter will be referred to as *Glomus intraradices*.

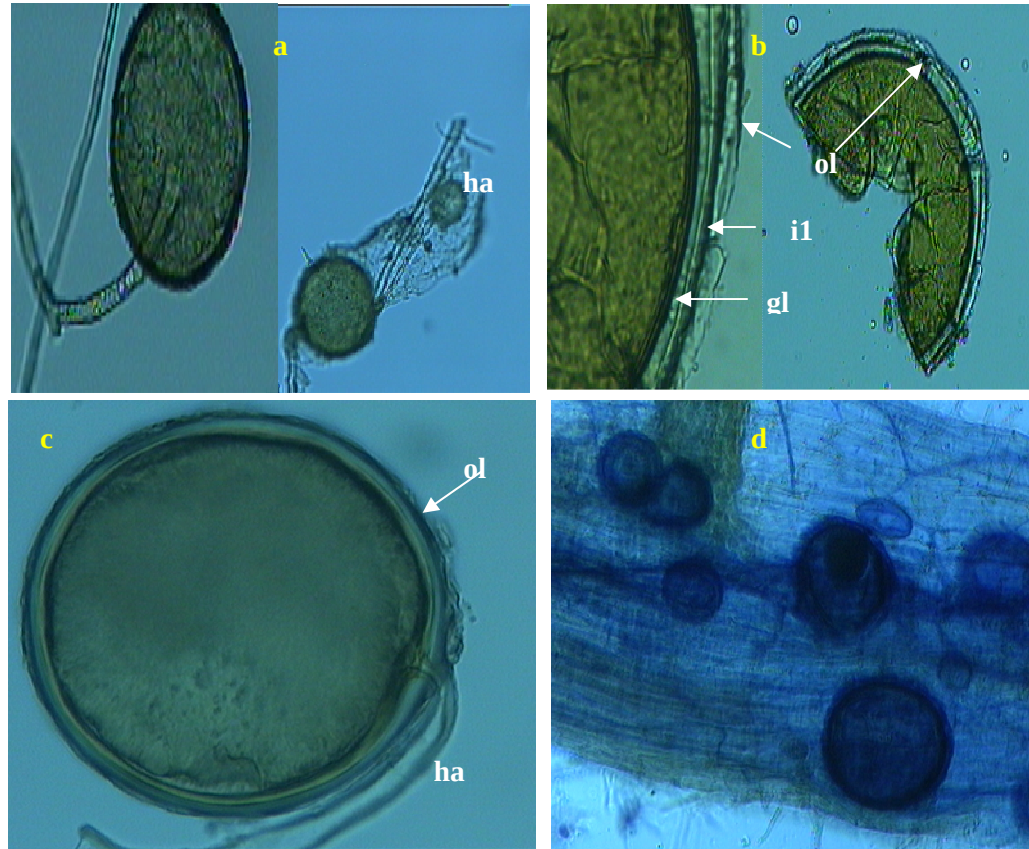


Figure 3.11: Microscope light of *Glomus intraradices*. a: Single spore with hyphal attachment (ha); b: crushed spore in Meltzer's staining reaction, ol: outer layer is colourless than others layers. c: wall layer in PVLG. ol: outer layer, d: spore in root formation.

The three type of AMF showed different distribution of spore diameter size. According to Seok Cho *et al.* (2006). The size of spores of AMF were very distinctive. Their diameter may vary from 10 μm , for *Glomus tenue*, to more than 1,000 μm , for some *Scutellospora* spp. The spores can vary in colour from hyaline (clear) to black and in surface texture from smooth to highly ornamented. *Glomus* forms spores on the ends of hyphae and the *Gigaspora* forms of inner membranous walls, germination shield a wall structure from which the germ tube can arise (Seok Cho *et al.* 2006) and bulbous sporogenous cell (INVAM).

The experiment was carried out to analyse the collection of AM fungi from Indonesia through investigation of physiological morphological characters and molecular biological techniques. The result obtained showed the importance of

exploring and exploiting the natural diversity of AM fungi, as a starting point to formulate inoculants to be applied as inoculums for the optimized plant growth.

3.3.2 Molecular identification of arbuscular mycorrhiza

Molecular characterization of AM fungal spores was performed by partial SSU and ITS rDNA sequence analyses for both types of spores morphologically identified as *Gigaspora rosea* (M1) and *Gigaspora albida* (M3).

SSU region amplification and sequencing

DNA extracts from single spores were amplified directly using NS31/AM1 primer pair. Different concentrations of DNA extracts were used. The result is shown in the Figure below.



Figure 3.12: Electrophoresis profile of *Gigaspora albida* PCR amplification products using a pair of primer AM31 and NS1, PCR product from 2.5 µl of DNA extract obtained from each single spore extraction (lane 1-5), 1 µl of DNA extract (6-10) and 1 µl of dilution 1:10 DNA extract (11-15).

Figure 3.12. showed that the PCR product obtained with a lower concentration of DNA extract have a better quality than the ones at higher concentration. For this reason 1 µl of dilution 1:10 DNA extract will be used in the subsequent amplifications. This could be caused by trace amounts of agents like

sodium hydroxide, which were used in DNA extraction procedures and strongly inhibit *Taq*DNA Polymerase.

The SSU region of both *Gigaspora* isolates (M1 and M3) from single spores was successfully amplified by using NS31/AM1 primer pair. In samples of both *Gigaspora* species, PCR products showed fragments with a size about 550 bp (Figure 3.13). Sequence analyses revealed that sequences had a size, which ranged between 545 and 546 bp.

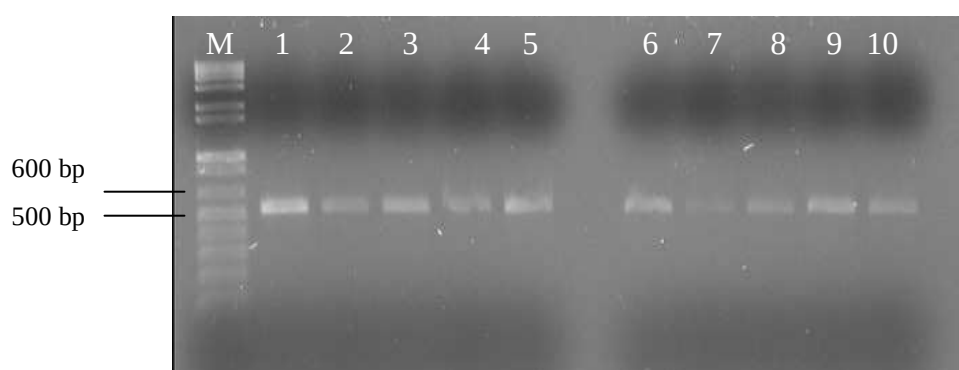


Figure 3.13: Electrophoresis profile of PCR products from amplification of *Gigaspora albida* (lane1-5) and *Gigaspora rosea* (lane6-10).

All SSU sequences from single spores of *Gigaspora albida* and *Gigaspora rosea* showed a high degree of similarity. From the alignment of the SSU sequences, both *Gigaspora* species showed 98% degree of similarity. Single spores from both *Gigaspora albida* and *Gigaspora rosea* contained SSU sequences, which showed differences in base substitution principally between 380 bp to 390 bp, and 410 to 470 bp, respectively. Single base substitution were found in all partial SSU rDNA sequences

Partial SSU rDNA sequences obtained in this work were aligned, using the ClustalW program from EMBL-EBI (Chenna *et al.* 2003), with sequences of Gigasporaceae available in GenBank. After alignment a phylogram was generated by TREECON for Windows software with the Neighbour-Joining method by using the Kimura-2 parameter (van de Peer and De Watcher, 1994). The confidence of branching was assessed using 1000 bootstrap resamplings

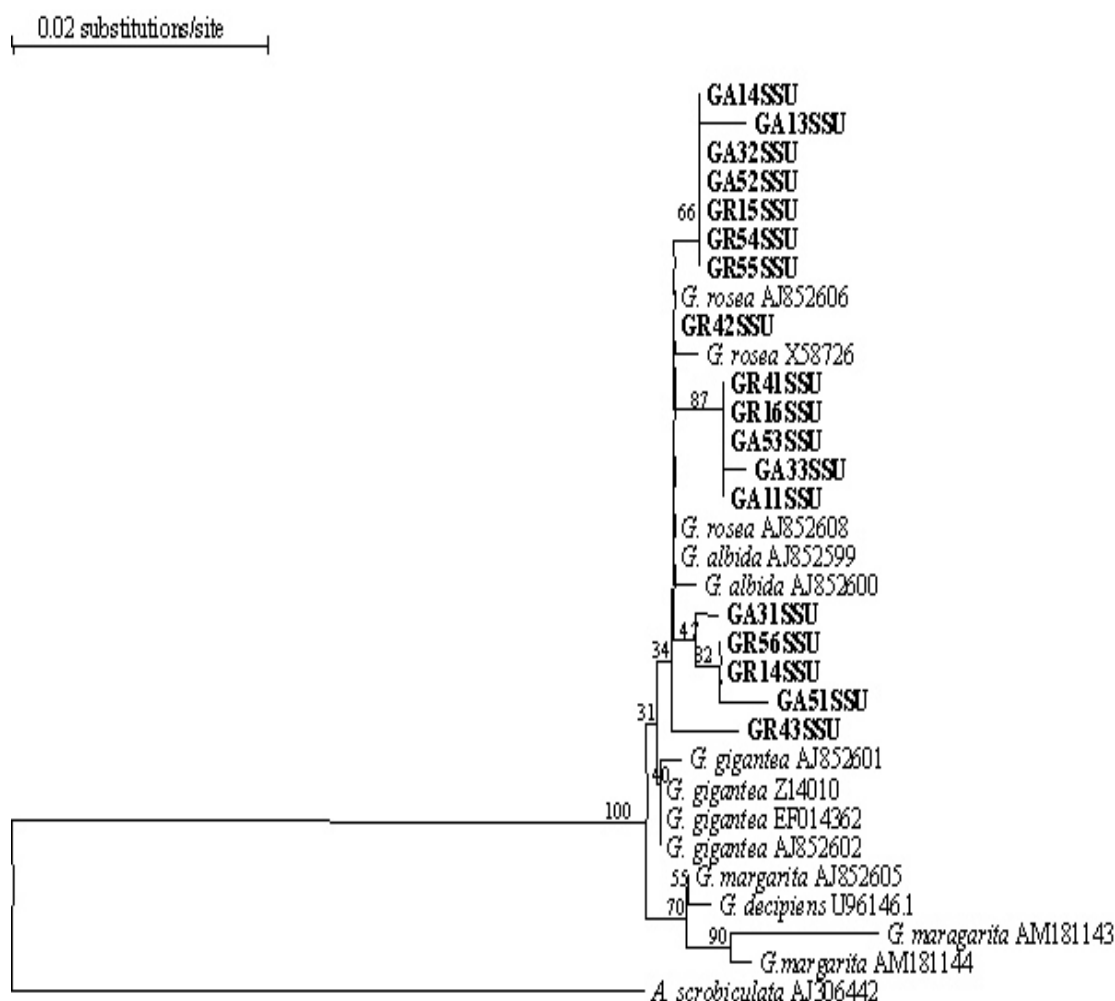


Figure 3.14: Neighbour-joining phylogenetic tree of Gigasporaceae, focusing on *Gigaspora* genus, obtained from the alignment of partial SSU rDNA sequences. Bootstrap values (out of 1000) are shown when they exceed 30%. *Acaulospora scrobiculata* was used as outgroup. Sequences obtained from M1 (GR sequences) and M3 (GA sequences) isolates are in bold.

Partial SSU rDNA sequences of the AMF (isolates M1 and M3), morphologically identified as *G. rosea* and *G. albida*, respectively, formed three clades (66, 87, 47 bootstap values), all containing sequences of both *G. albida* (GA sequences) and *G. rosea* (GR sequences). GR43SSU and GR42SSU did not enter in the three clades, but were contained in the main clade containing *G. rosea* and *G.*

albida sequences from GenBank. *G. gigantea*, *G. margarita* and *G. decipiens* formed separated branches.

ITS region amplification and sequencing.

G. rosea and *G. albida* DNA extracts from single spores were amplified by using a semi-nested protocol (ITS1F/ITS4-ITS1-ITS4 primers pairs) (Figure 3.15). PCR reactions were performed on 5 single spores for both *Gigaspora* species but only PCR products from 3 single spores/species gave enough DNA for cloning.

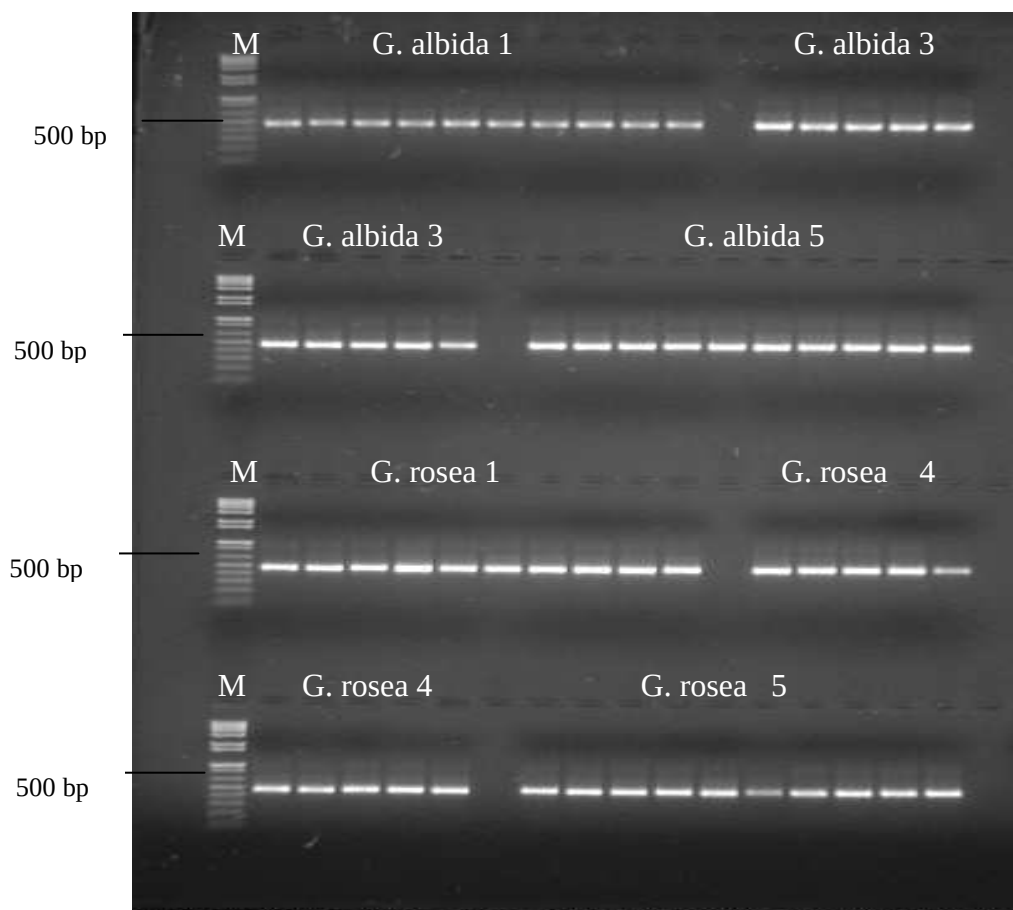


Figure 3.15: Electrophoresis profile of *Gigaspora albida* spore 1 *G. albida* spore 3 *G. albida* spore 5 *G. rosea* spore 1 (*G. rosea* spore 4 and *G. rosea* spore 5 (clone 1/10) from PCR nested ITS colony PCR product.

Phylogenetic tree showing the relationships between *G. rosea* and other species, based on ITS sequences. The tree is rooted with *A. denticulata* AJ239115. Bootstrap values are indicated at the nodes.

Species and Accession Numbers:

- G. rosea* AJ410746
- GR16ITS**
- GA54ITS**
- G. rosea* AF004701
- GR42ITS**
- GA51ITS**
- GR410ITS**
- G. rosea* AJ410749
- GR514ITS**
- GA56ITS**
- GR44ITS**
- GA312ITS**
- G. rosea* AJ504639
- GR51ITS**
- GA31ITS**
- GR53ITS**
- GR46ITS**
- GR17ITS**
- G. gigantea* AJ410751
- G. gigantea* AF004685
- G. gigantea* AJ410752
- GR12ITS**
- GA11ITS**
- GR18ITS**
- G. rosea* AF004700
- GR512ITS**
- G. rosea* AF004696
- G. rosea* AJ410745
- GR11ITS**
- GA310ITS**
- G. rosea* AJ410748
- GR41ITS**
- GA32ITS**
- G. rosea* AJ410750
- GA510ITS**
- GA39ITS**
- GA12ITS**
- G. albida* AF004704
- G. albida* AF004707
- G. albida* AF004703
- GA113ITS**
- GA15ITS**
- GR54ITS**
- G. rosea* AF004699
- GA59ITS**
- GA13ITS**
- G. margarita* AY035643
- G. decipiens* AJ239119
- G. margarita* AF162465
- G. margarita* AJ410741
- G. margarita* AB048627
- G. margarita* AF162460
- A. denticulata* AJ239115

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Multiple sequences alignment of *G. rosea* and *G. albida* sequences showed that the interspecific and intraspecific variation in the ITS region was very high. ITS1/ITS 4 semi-nested PCR produced fragments which ranged about 482 bp to 492 bp and 483 bp to 496 bp from spores of *G. albida* and *G. rosea*, respectively. A neighbour-joining phylogenetic analysis was performed by using the alignment of ITS sequences of Gigasporaceae from GenBank and ITS sequences from M1 and M3 isolates. A very high intraspecific variation was found also with ITS region and sequences from clones of both species clustered together. Two main clades, containing *G. rosea* and *G. albida* sequences were obtained. *G. margarita* sequences clustered in a separated clade (39% bootstrap). *G. gigantea* sequences formed a separated branch (89% bootstrap) inside *G. rosea*/*G. albida* main branch. Most ITS sequences derived from *G. rosea* spores (isolate M1) (12 out of 15) clustered in the branch where most of the *G. rosea* sequences derived from GenBank clustered. However *G. rosea* sequences were present also in the cluster containing *G. albida* sequences from GenBank. Sequences derived from *G. albida* (isolate M3) were present in both the clades. A large heterogeneity was present in the ITS region sequences also within the same single spore. The heterogeneity measured in this study was so large that no one out of thirty sequences from single spores of *G. albida* and *G. rosea* were identical. Identity degree between samples of the two *Gigaspora* morphotype was between 91-99 %. An high heterogeneity of ITS region within one single spores was also reported by Sanders *et al.* (1995), Lloyd-Macgilp *et al.* (1996); Clapp *et al.* (1995) and Antonioli *et al.* (2000).

In conclusion SSU and ITS sequence analyses confirmed that the AMF studied belonged to the genus *Gigaspora*, but our molecular data showed that it was not possible to distinguish between *Gigaspora albida* and *Gigaspora rosea*. Moreover the ITS sequence analysis showed a very high degree of heterogeneity, even if spore morphological analyses allowed the identification of the two species on the basis of spore size, hyphal wall layers and colour

Table 3.1 Percentage identical sequence ITS of *Gigaspora albida* as a compare with the sequences from the gene bank using Blast program (Zhang *et al.* 2000), from the NCBI home page

Sequences AMF in this identification	Sequences from data base	Accession no from data base	Identity percentage compare with data base sequences
GA11ITS	<i>Gigaspora albida</i>	AF004703.1	93
GA12ITS	<i>Gigaspora albida</i>	AF004703.1	96
GA13ITS	<i>Gigaspora albida</i>	AF004703.1	97
GA15ITS	<i>Gigaspora albida</i>	AF004703.1	97
GA113ITS	<i>Gigaspora albida</i>	AF004703.1	97
GA31ITS	<i>Gigaspora albida</i>	AF004702.1	94
GA32ITS	<i>Gigaspora albida</i>	AF004703.1	95
GA39ITS	<i>Gigaspora albida</i>	AF004703.1	95
GA310ITS	<i>Gigaspora albida</i>	AF004703.1	96
GA312ITS	<i>Gigaspora albida</i>	AF004702.1	96
GA51ITS	<i>Gigaspora albida</i>	AF004702.1	96
GA54ITS	<i>Gigaspora albida</i>	AF004702.1	95
GA56ITS	<i>Gigaspora albida</i>	AF004702.1	95
GA59ITS	<i>Gigaspora albida</i>	AF004703.1	98
GA510ITS	<i>Gigaspora albida</i>	AF004703.1	95

Table 3.2: Percentage identical sequence ITS of *Gigaspora rosea* as a compare with the sequences from the gene bank using Blast program (Zhang *et al.* 2000), from the NCBI home page.

Sequences AMF in this identification	Sequences from data base	Accession no from data base	Identity percentage compare with data base sequences
GR11ITS	<i>Gigaspora rosea</i>	AJ410747.1	97
GR12ITS	<i>Gigaspora rosea</i>	AJ410746.1	96
GR16ITS	<i>Gigaspora rosea</i>	AJ504639.1	97
GR17ITS	<i>Gigaspora rosea</i>	AJ504639.1	95
GR18ITS	<i>Gigaspora rosea</i>	AJ410743.1	98
GR41ITS	<i>Gigaspora rosea</i>	AJ410748.1	100
GR42ITS	<i>Gigaspora rosea</i>	AJ410749.1	99
GR44ITS	<i>Gigaspora rosea</i>	AJ504639.1	94
GR46ITS	<i>Gigaspora rosea</i>	AJ504639.1	94
GR410ITS	<i>Gigaspora rosea</i>	AJ410749.1	98
GR51ITS	<i>Gigaspora rosea</i>	AJ504639.1	97
GR53ITS	<i>Gigaspora rosea</i>	AJ504639.1	94
GR54ITS	<i>Gigaspora rosea</i>	AF004699.1	98
GR514ITS	<i>Gigaspora rosea</i>	AJ504639.1	97
GR512ITS	<i>Gigaspora rosea</i>	AJ410743.1	99

Restriction Analysis of PCR amplified ITS rDNA

Both of *Gigaspora* species were analysed comparing the restriction fragment lengths of the PCR amplified ITS rDNA using the molecular weight marker 1 kb. Figure 3.17 and 3.18 showed the restriction patterns generated with the enzyme *Mbo*I for *G. albida* and *G. rosea*.

Table. 3.3: Lengths of restriction fragments of ITS PCR products from arbuscular mycorrhiza

AMF	The range fragment length(s) (bp)	Number of fragment	Variant of fragment
<i>Gigaspora albida</i> 1	89±2 169±1 194±2	3	4
<i>Gigaspora albida</i> 3	88±2 169±1 6±3	3	4
<i>Gigaspora albida</i> 5	92±2 168±1 197±2	3	4
<i>Gigaspora rosea</i> 1	92±1 168±1 195±3	3	4
<i>Gigaspora rosea</i> 3	91±3 168±1 198±3	3	5
<i>Gigaspora rosea</i> 5	91±2 168±1 196±3	3	3

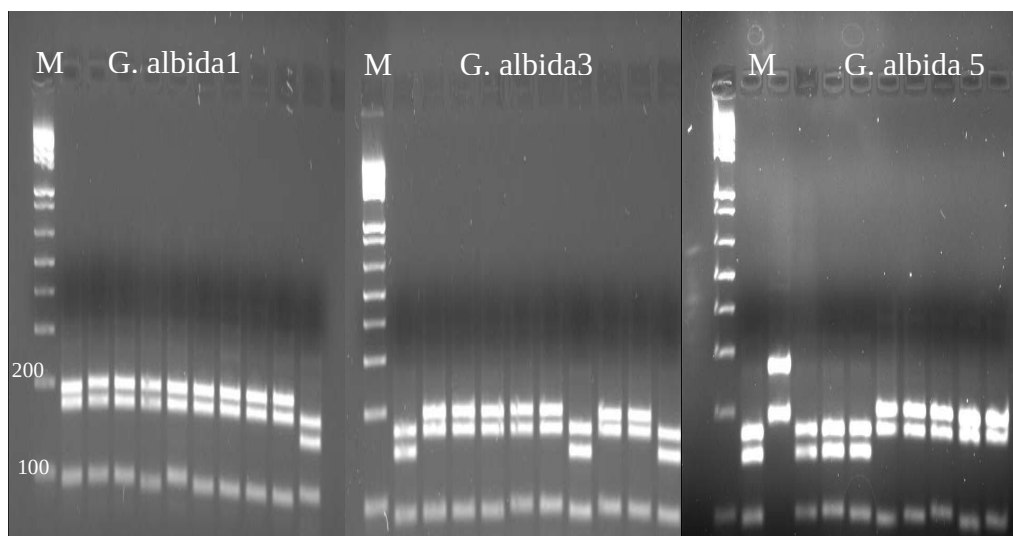


Figure 3.17: Restriction fragment patterns of ITS fragments from 3 single spore of *Gigaspora albida*. Colony PCR products were digested with the enzyme *mbol* and run on a 2% agarose gel, Lane M, molecular size marker.

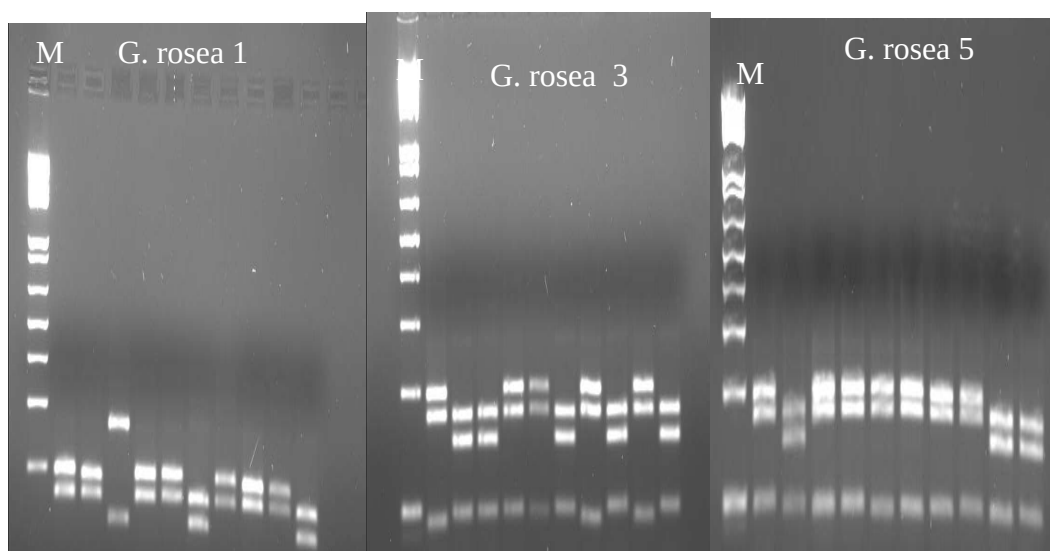


Figure 3.18: Restriction fragment patterns of ITS fragments from 3 single spore of *Gigaspora rosea*. Colony PCR products were digested with the enzyme *mbol* and run on a 2% agarose gel, Lane M, molecular size marker.

Restriction fragment length polymorphism (T-RFLP) analysis is a polymerase chain reaction (PCR)-fingerprinting method that is commonly used for comparative microbial community analysis. The method is rapid, highly reproducible, and often yields a higher number of operational taxonomic units than other The method can be

used to analyze communities of bacteria, fungi, other phylogenetic groups or subgroups (Thies 2007).

ITS PCR products from single spores were analysed by RFLP, using *MboI* as restriction enzyme. The RFLP analysis was not sufficient to distinguish morphospecies of the *Gigaspora* tested. The restriction pattern calculated on the sequences did not show significant differences between the studied species (Table 3.3) But the pattern of the fragments generated with the enzyme *MboI* was different from what was expected, because there were variant bands in different amplification of the same DNA extract as shown in Figure 3.17 and 3.18 with a fragment ranged from 90 to 200 bp. Redecker *et al.* (1997) reported that the patterns of fragment size generated with the enzyme *MboI* is the same for *G. albida* and *G. rosea* with fragments of size 100, 175 and 200 bp. These results are quite similar to what we have obtained; in fact both *Gigaspora* species showed 3 restriction fragments with a range size of about 90, 168 and 197 bp.

3.4 Micropropagation of olive

Micropropagation is a technique of increasing importance for the mass multiplication of quality and disease free plants of many commercial crops. Today *in vitro* reproduction of olive which is one of the most important fruit trees in the Mediterranean area is still in progressing., some experiments have been carried out in order to in attempt to improve the quality and speed up the production of micropropagated olive *in vitro*;

3.4.1 Effect dikegulac in micropropagation of olive

As shown in the Table 3.4 the cvs Canino, Frantoio and Moraiolo showed an increased of shoot proliferation. Media supplemented with dikegulac at concentration of 66.7 μM was gave the best result because it showed higher number of shoots and nodes without reducing shoot length. The highest number of shoots was obtained at dikegulac concentration of 100.5 μM , with more than four shoots per explants but with a severe reduction in length. Increasing dikegulac concentration up to 133.4 μM

resulted in a considerable reduction of the number of shoots as well as nodes per explant.

Table 3.4: Effect of dikegulac concentrations on shoot proliferation, shoot length and total nodes per explant after a 40-day culture on proliferation media (ROM, with 0.45 zeatin and 3.6% mannitol and 0.7% plant agar) supplemented with different concentrations of dikegulac.

Cultivar	Concentration of dikegulac (μM)	Number Shoots per explant	Length of shoots (cm)	Number nodes per explants	Survival (%)
Canino	0.0	$1.4 \pm 0.11\text{cd}$	$5.0 \pm 0.34\text{a}$	$4.9 \pm 0.32\text{b}$	100a
	16.9	$1.7 \pm 0.15\text{bc}$	$5.6 \pm 0.38\text{a}$	$5.2 \pm 0.27\text{b}$	100a
	33.8	$2.2 \pm 0.16\text{bc}$	$5.5 \pm 0.29\text{a}$	$10.1 \pm 0.52\text{a}$	100a
	66.7	$2.5 \pm 0.19\text{b}$	$4.4 \pm 0.35\text{a}$	$9.9 \pm 0.74\text{a}$	100a
	100.5	$4.4 \pm 0.45\text{a}$	$2.4 \pm 0.21\text{b}$	$10.0 \pm 0.58\text{a}$	100a
	133.4	$1.0 \pm 0.13\text{d}$	$1.5 \pm 0.38\text{b}$	$2.1 \pm 0.31\text{c}$	$55 \pm 3.5\text{b}$
Frantoio	0.0	$1.3 \pm 0.11\text{cd}$	$4.8 \pm 0.35\text{a}$	$4.8 \pm 0.26\text{d}$	100a
	16.9	$1.6 \pm 0.17\text{c}$	$5.2 \pm 0.26\text{a}$	$6.5 \pm 0.60\text{cd}$	100a
	33.8	$2.2 \pm 0.17\text{b}$	$5.9 \pm 0.40\text{a}$	$7.4 \pm 0.58\text{bc}$	100a
	66.7	$2.7 \pm 0.24\text{b}$	$4.9 \pm 0.23\text{a}$	$8.9 \pm 0.66\text{a}$	100a
	100.5	$4.7 \pm 0.35\text{a}$	$2.2 \pm 0.21\text{b}$	$10.0 \pm 0.60\text{a}$	100a
	133.4	$1.0 \pm 0.12\text{d}$	$2.3 \pm 0.40\text{b}$	$1.0 \pm 0.09\text{e}$	$50 \pm 3.3\text{b}$
Moraiolo	0.0	$1.7 \pm 0.09\text{c}$	$3.8 \pm 0.33\text{a}$	$3.6 \pm 0.33\text{bc}$	100a
	16.9	$2.1 \pm 0.18\text{bc}$	$4.5 \pm 0.24\text{a}$	$6.0 \pm 0.66\text{ab}$	100a
	33.8	$2.7 \pm 0.28\text{bc}$	$4.8 \pm 0.41\text{a}$	$6.8 \pm 0.80\text{a}$	100a
	66.7	$3.1 \pm 0.26\text{b}$	$4.1 \pm 0.23\text{a}$	$7.2 \pm 0.64\text{a}$	100a
	100.5	$4.2 \pm 0.46\text{a}$	$3.1 \pm 0.41\text{b}$	$8.6 \pm 1.10\text{a}$	100a
	133.4	$4.1 \pm 0.11\text{d}$	$3.2 \pm 0.45\text{b}$	$1.1 \pm 0.11\text{c}$	$40 \pm 3.4\text{b}$
Rosciola	0.0	$1.8 \pm 0.22\text{a}$	$4.2 \pm 0.41\text{a}$	$4.6 \pm 0.27\text{a}$	100a
	16.9	$1.4 \pm 0.11\text{ab}$	$1.9 \pm 0.26\text{b}$	$3.2 \pm 0.13\text{b}$	100a
	33.8	$1.0 \pm 0.17\text{b}$	$1.5 \pm 0.21\text{c}$	$0 \pm 0.10\text{c}$	$60 \pm 2.9\text{b}$
	66.7	$1.0 \pm 0.11\text{b}$	$1.0 \pm 0.09\text{d}$	$1.0 \pm 0.07\text{cd}$	$40 \pm 2.6\text{bc}$
	100.5	$1.0 \pm 0.09\text{b}$	$0.8 \pm 0.09\text{d}$	$1.0 \pm 0.05\text{cd}$	$25 \pm 2.5\text{c}$
	133.4	$0.0 \pm 0.0\text{c}$	$0.0 \pm 0.0\text{d}$	$0.0 \pm 0.0\text{d}$	0c
Piantone di moiano	0.0	$1.7 \pm 0.26\text{a}$	$4.0 \pm 0.40\text{a}$	$2.6 \pm 0.26\text{a}$	100a
	16.9	$2.0 \pm 0.26\text{a}$	$4.1 \pm 0.60\text{a}$	$2.0 \pm 0.15\text{a}$	100a
	33.8	$1.4 \pm 0.16\text{a}$	$2.7 \pm 0.50\text{b}$	$1.4 \pm 0.71\text{b}$	100a
	66.7	$1.0 \pm 0.17\text{b}$	$2.5 \pm 0.65\text{b}$	$1.4 \pm 0.45\text{b}$	$50 \pm 2.3\text{b}$
	100.5	$1.0 \pm 0.13\text{b}$	$1.0 \pm 0.75\text{c}$	$1.0 \pm 0.09\text{b}$	$40 \pm 2.6\text{bc}$
	133.4	$1.0 \pm 0.10\text{b}$	$1.0 \pm 0.11\text{c}$	$1.0 \pm 0.05\text{b}$	$20 \pm 2.1\text{c}$

Note: Data represent means values with different letters within a column and a cultivar, indicate significant differences (P 0.05)

Supplying dikegulac to Rosciola and Piantone di Moiano had almost no effect or a negative effect on shoot formation and at concentration above 33.8 μM explant survival was reduced. The combination of zeatin and dikegulac has shown a further

improvement in shoot multiplication of the cultivars analysed in this experiment. The effect of dikegulac in apical dominance reduction differs in various olive cultivars and the optimum concentration needs to be determined for each cultivar.

Dikegulac reduces apical dominance and promotes lateral branching and lower-bud formation in some plants (Bocion *et al.* 1975; Sachs *et al.* 1975; Norcini *et al.* 1994; Das *et al.* 2006). Dikegulac was also tested in field-grown adult olives for its capacity to increase fruit set as a result of the temporary plant growth reduction, and increase in the number of short shoots (Rugini and Pannelli 1993). The effect of dikegulac in apical dominance reduction differs in various olive cultivars and the optimum concentration needs to be determined for each cultivar. The use of dikegulac may contribute to an improvement of the olive clonal propagation from nodal segments.

3.4.2 Effect of three different light conditions in the development of roots for the cv Canino *wt* and transgenic *rolABC* Canino

The light influence on the rooting performance of cv Canino WT and transgenic Canino *rolABC* *eco15-5* during the rooting phase was recorded.

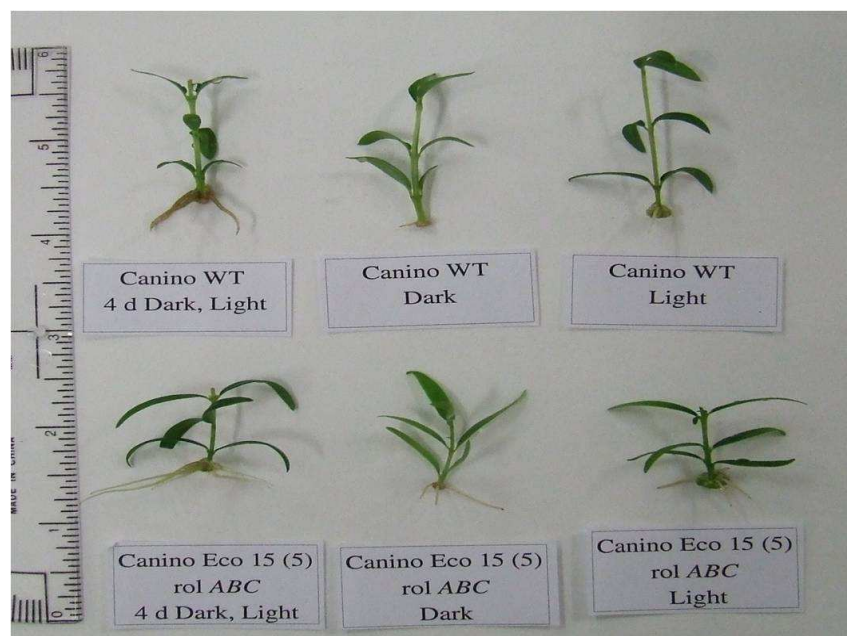


Figure 4.19: Effect of light incubation in number of roots Canino WT and transgenic Canino *rolABC* Eco15-5 in *in vitro* culture

The rooting plants were put in the rooting medium supplemented with Putrescine and IBA, in three different light condition: L1 plants put in dark for four days and transferred to the light in growth chamber; L2 plants were always in complete darkness from the beginning of rooting phase, and L3 plants were always in the light during all rooting process.

As shown in Table 3.5 the results gave a significant root number with the transgenic cv Canino *rolABC*, the highest number of roots than other treatments, and the best result for root length was reached with Canino WT.

Table 3.5: Effect of the light condition treatments on rooting induction in *in vitro* grown plants of olive. The temperature under the three trials was maintained constant value of 25 ± 1 °C.

Culture condition	% rooting	Number of root	Root length mm
dark for initial 4 days and than light			
Canino wild type	100	2.7 ± 0.38^{bc}	16.5 ± 0.77^a
Canino <i>rolABC</i>	80	4.2 ± 0.80^a	5.6 ± 0.90^b
dark treatment			
Canino wild type	80	2.3 ± 0.38^{bc}	1.98 ± 0.77^b
Canino <i>rolABC</i>	100	2.6 ± 0.80^{bc}	3.9 ± 0.90^b
light treatment			
Canino wild type	100	2.0 ± 0.38^{bc}	4.97 ± 0.77^b
Canino <i>rolABC</i>	100	3.6 ± 0.80^{ab}	4.3 ± 0.90^b

Note: Different letter indicate statistically different values per $P=0.05$ for each parameter.

Figure 3.20, demonstrated L1 treatment showed better results for number of roots compared to L2, and L3 treatments, with representative means of 2.74, 2.25 and 2 for Canino WT respectively and 4.2, 2.6 and 3.6 for Canino transgenic *rolABC* Eco15-5 respectively. As shown in Figure 3.21, the root length showed significant different results for the 3 different light treatments in the two type olive cultivars. The Canino WT under L1 treatment gave the longest roots compared with the other treatments and transgenic Canino of the root with longest root compare with other treatment and Canino transgenic.

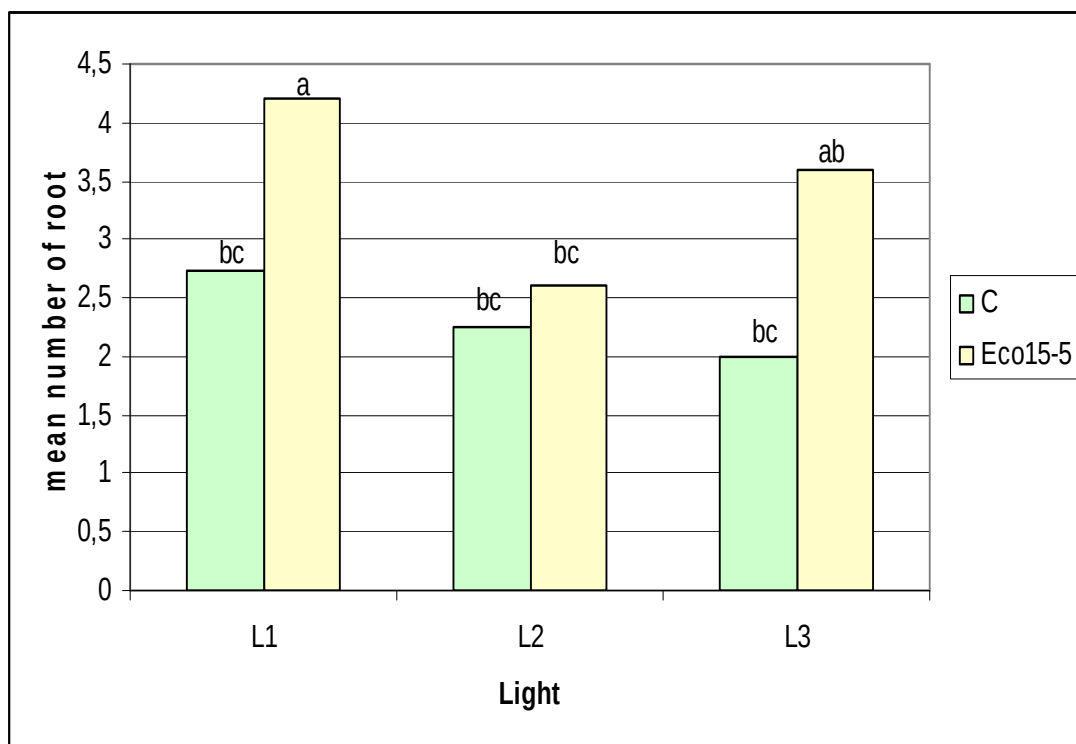


Figure 3.20: Effect of light culture conditions on number of roots t of Canino WT and transgenic Canino *rolABC* Eco15-5 in *in vitro* culture. . L1: incubated in 4 days dark than to light until the end of rooting phase, L2: always maintained in the dark. L3: always was maintained in the light. Different letters indicate statistically different values per $P=0.05$.

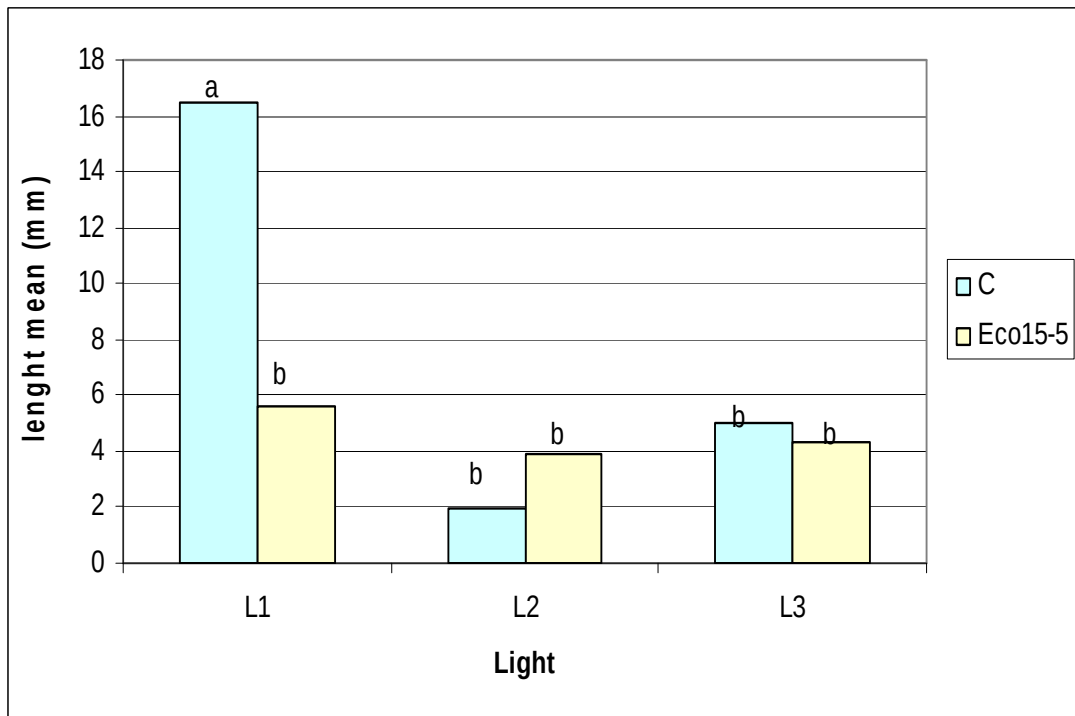


Figure 3.21: Effect of the light culture condition on root length of Canino WT and transgenic Canino *rolABC* Eco15-5 in *in vitro* culture. L1: incubated in 4 days dark than to light until the end of rooting phase, L2: always maintained in the dark. L3: always was maintained in the light. Different letters indicate statistically different values per $P=0.05$.

According to Rugini and Fedeli (1999) and Rugini *et.al.* (1987) adventitious root induction in olive is mainly depended on the shoot quality and environment. Rugini also suggested that the initial dark condition during the rooting phase caused an increase in rooting and rooting growth. The result of the two olive cultivars reported here, which showed the best rooting number and length under 4 days in dark, was used as the rooting procedure for the screening of AMF.

3.4.3 Multiplication and rooting in vitro.

For screening AMF on micropropagated olive plants, 6 cultivars were micropropagated for 40 days in agar solidified medium, containing Olive Medium (OM) macro, microelements and vitamins (Rugini 1984), supplemented with filter sterilized zeatin after autoclaving. For each 500 ml jar containing 70 ml of medium were planted 8-10 explants (Figure 3.22)



Figure 3.22: Micropropagation olive plant of cv Moraiolo (left) and cv Canino (right)

The micropropagation olive has been done several times until the number of explants was enough for the screening with AMF. After the multiplication, explants were transferred to rooting medium enriched with Putrescine 160 mg/l and IBA 0.4mg/l (Figure 3.23).



Figure 3.23: Unrooted and rooted olive plant after 25 days, in stage 2 -4 node.

During the work on micropropagation, we observed that different types of bottle jars induced different growth habits. When they were cultivated in jars with a black lid, plants showed lower shoot branching in nodes than those grown in jars having the transparent lid. The number of shoots, nodes and plant height presented here were recorded after 40 days of culture, from seven plants from each of type per jar.

Table 3.6: Effect of lid jar type on shoot, length and nodes per explant after 40 days of subculture

Cultivar	Type of Lid	Height	Nodes per explants	Number of shoots
Canino	Transparent	5.3±0.67b	7.1±1.21c	4.1±2.79
	Black	7.1±0.70a	7.4±0.98b	1.0±1.29
Canino <i>rolABC</i>	Transparent	6.7±2.44b	10.7±1.98a	2.9±1.35
	Black	7.4±0.48a	8.9±0.69b	1.3±1.60

Note: Different letter indicate statistically different values per P=0.05 for each parameter

Different lids influenced the different quantity of light that enters into the bottle. We observed that the explants, which grew in jars with transparent lid, were healthy with high proliferation rate for both cultivars, but showed an opposite development for shoot height. Regarding shoot height, the highest result were showed by cv Canino transgenic followed by Canino WT, which were put in the dark lid (Figure 3.24.).



Figure 3.24: Lid jar effect on growth habit in olive under *in vitro* conditions. Arrow indicated a shoot branches in the nodes.

Light is a fundamental clue in life plants, playing a crucial role directly and directly in the regulation of plant development and growth (Morini and Muleo 2003). It is as well an important source of energy used by green plants for their four main mechanisms: photosynthesis, photomorphogenesis, photoperiodism and phototropism (Aphalo 2006; Morini and Muleo 2003).

Photosynthesis is the essential process in which plants convert light energy into chemical energy in the form of organic molecules, some of which also serve as building blocks for plant structure. Photomorphogenesis is the effect of light intensity and quality on plant growth and development, like germination and leaf, stem development. Some plants need a lot of light to germinate from their seeds. Lack of sufficient light in this process can often also result in plants that are lacking in normal colour due to a lack of chlorophyll being produced. The other category, which effect on the relative length of the daily light/dark period mainly on flowering, is called photoperiodism. The last one are phototropism is the directional growth of plant parts (leaves, stems, roots) in which the direction of bending is determined by the direction of the light source.

In our study we found that light quantity effected the development of shoots, length of shoots and nodes, therefore, light is one of critical factor that affects the developmental processes within plants *in vitro* culture beside plant material, nutrient and another room culture factor.

3.5 Effect of arbuscular mycorrhiza on micropropagated olive

Four different type AMF: M1, M2, M3 and M4 all native to Indonesia were used to study the influence of this symbiotic on olive micropropagated plants cultivar Moraiolo, Canino and three different transgenic *osmotin* Canino (clones AT17-2, At17-3, and At17-10) which were more resistant to fungi, and transgenic for *rolABC* Canino, which reduce growth and increase rooting capability. Observations have been made to plant height, number of leaves, root volume and shoot volume, leaf area and chlorophyll content on the leaves. Data were analysed statistically using the R program. *In vitro* rooted plantlets were cleaned from the agar media and transferred to a sterile pot containing mixed sand and soil (1:1) by volume and inoculated with different type of AMF in order to evaluate this effect on plant survival and growth.

After 4 weeks, the percentage of survival ranged from 40% to 100% (Fig. 4.23). The survival varied according to type of AMF and cultivar of olive. The lowest survival was observed among transgenic plants. It seems that AMF helped the plantlets to resist the environmental stress during transplanting them from axenic conditions to normal cultivation in pots.

The survival of plants are an important factor for *post vitro* establishment of micropropagated plants, since the most critical step in micropropagation process is transfer of *in vitro* plantlets to *ex vitro* condition. Our result confirmed what was reported before by several authors on the other micropropagated species, such as apple and plum (Fortuna *et al.* 1996), artichoke (Morone Fortunato, *et al.*, 2005) and Origanum (Morone Fortunato and Avato, 2008).

It is well known that the natural growth and development depend on formation of arbuscular fungi in many woody plants and trees. Therefore inoculations with AMF may help to overcome problems associated with micropropagation of woody and fruit trees as stated by Taylor and Harrier (2003).

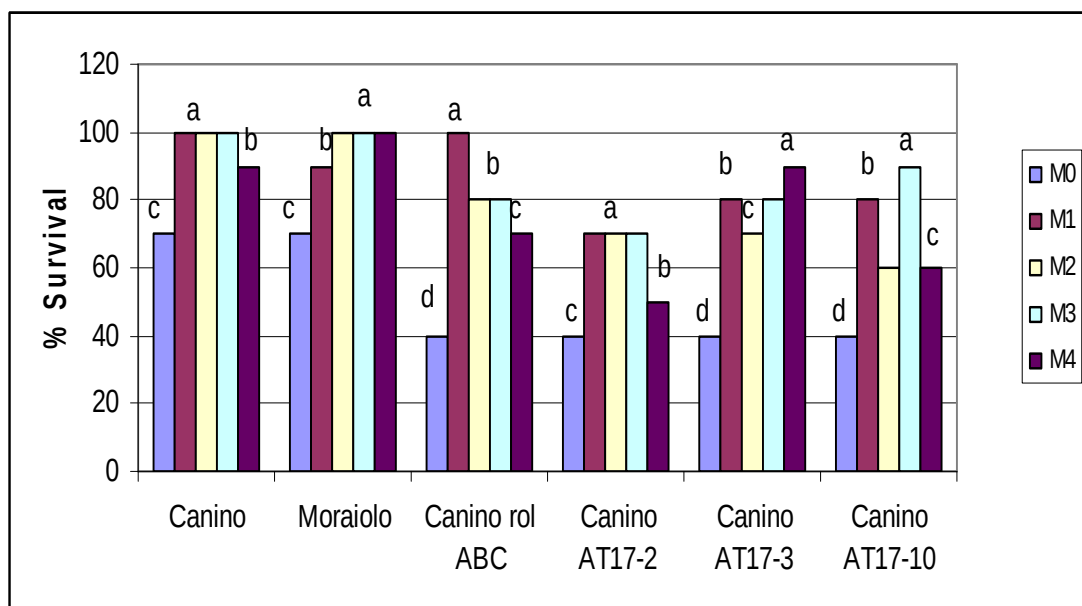


Figure 3.25: Survival rate (%) of olive plant after 4 weeks acclimatization stage; different letter indicate statistically different values per P= 0.05

The growth development of olive plantlet cv Moraiolo and Canino wt which treated with AMF showed higher length of shoot and number leaves (Figure 3.26) than untreated with AMF.

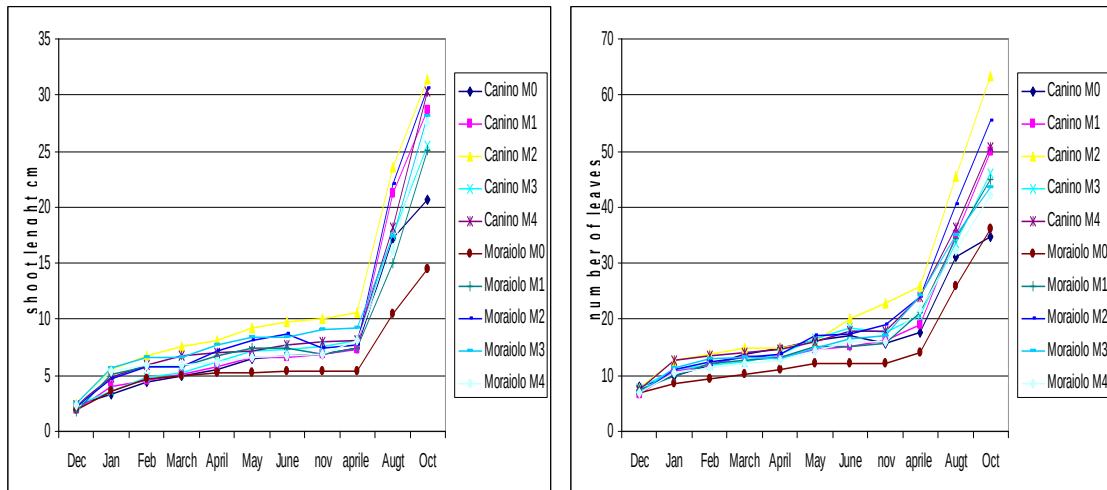


Figure 3.26: The length of shoots and number of leaves of Canino and Moraiolo plants, with different application of arbuscular mycorrhiza (M1, M2, M3, M4) in comparison with untreated (K).

The plants have been potted to poor substrate (composed of soil and sand as 1:1 volume) for 15 months, in the attempt of evaluating better all the effect of AMF to olive cultivars considering the adverse condition determined by this substrate composition. In fact, all the plantlets grew very slowly, but those treated with AMF had better adaptation which showing better growth development than non inoculated plant, although several plants were lost, mainly due to the improper substrate. After 15 months, when the plantlets were transferred to 1,5 l pots with a suitable substrate, they grew quickly.

The olive plant, treated and not-treated with AMF, showed differences on growth development than untreated ones, liked showed in micropropagated Moraiolo (Figure 3.27.) and Canino (Figure 3.28), which were treated with different types of AMF.



Figure 3.27: Moraiolo plants, with different application of arbuscular mycorrhiza (M1, M2, M3, M4) in comparison with untreated (K).



Figure 3.28: Canino plants with different application of arbuscular mycorrhiza (M1, M2, M3, M4) in comparison with control.

The percentage of roots colonized of all type AMF varied from 3.4 to 70.9 % according to type of AMF and olive cultivar. The highest colonization was observed in cv Canino WT with AMF M4. (Table 3.6). These results confirm what was reported by Calvente *et al.* (2004) in others cultivars of olive plantlets with other type of AMF; in fact they observed big variation of colonization according not only with olive cultivar but also with different type of AMF.

Our result showed that both cv Canino treated with AMF resulted in higher growth development compared with the corresponding transgenic olive plants. The application of AMF increased shoot length for about 1,5-2 times according to cultivar, however less than the length reported by Binet *et al* (2007), which observed 3 fold increase. Our results are similar to those reported by Calvente *et al* (2004) in olive plantlets cv Leccino, where they observed small difference between control and treated plants with mycorrhiza. In addition, they noted that the isolate of native AMF were more effective in improving growth of both olive cv Leccino and Arbequina plantlets than isolate not native.

Table 3.7: Data Average $\pm$ SE, of the root shoot fresh weight, root length (cm), shoot (cm) and root, shoot volume (mm³) and % of root colonized.

Cultivar	AMF	weight (g)	root (cm)	shoot (cm)	root (mm3)	shoot (mm3)	%AM
Canino AT17-3	M0	2.0 $\pm$ 0.2	24 $\pm$ 1.4	14.5 $\pm$ 2.1	2226.6 $\pm$ 346.9	2207.8 $\pm$ 4906.3	0
	M1	5.5 $\pm$ 3.6	30 $\pm$ 9.9	28.5 $\pm$ 10.6	3189.1 $\pm$ 1040.8	4906.3 $\pm$ 1387.7	10.8
	M2	3.6 $\pm$ 0.6	32 $\pm$ 5.7	20.5 $\pm$ 2.1	2698.4 $\pm$ 346.9	3434.4 $\pm$ 693.8	5,9
	M3	2.9 $\pm$ 0.1	20.5 $\pm$ 0.7	14.8 $\pm$ 3.2	2453.1 $\pm$ 0.00	3557 $\pm$ 173.5	19,0
	M4	6.0 $\pm$ 0.8	30 $\pm$ 2.8	20.8 $\pm$ 2.1	3189.1 $\pm$ 346.9	6132.8 $\pm$ 1040.8	3,7
Canino AT17-2	M0	-	-	-	-	-	-
	M1	3.7 $\pm$ 1.8	26.7 $\pm$ 2.1	18.7 $\pm$ 2.9	1390.1 $\pm$ 141.6	1962.5 $\pm$ 490.6	12,1
	M2	8.0 $\pm$ 2.4	33 $\pm$ 8.2	29.0 $\pm$ 1.7	2943.7 $\pm$ 1982.8	3761.46 $\pm$ 849.8	5,9
	M3	4.1 $\pm$ 0.6	32 $\pm$ 15.9	22.0 $\pm$ 5.2	2126.0 $\pm$ 861.5	2128.0 $\pm$ 282.3	19,0
	M4	6.4 $\pm$ 1.5	36.7 $\pm$ 1.1	30.4 $\pm$ 2.4	2671.2 $\pm$ 249.8	4633.7 $\pm$ 377.7	3,7
Canino AT17-10	M0	-	-	-	-	-	-
	M1	4.2 $\pm$ 1.1	31.5 $\pm$ 2.1	26.5 $\pm$ 3.5	2453.1 $\pm$ 2081.5	3434.4 $\pm$ 1387.7	11,5
	M2	-	-	-	-	-	-
	M3	-	-	-	-	-	-
	M4	4.4 $\pm$ 1.1	38.5 $\pm$ 2.1	26.5 $\pm$ 3.5	3434.3 $\pm$ 2081.5	3925.0 $\pm$ 1387.7	3,4
Canino rol ABC	M0	-	-	-	-	-	-
	M1	-	-	-	-	-	-
	M2	1.8 $\pm$ 0.8	26.0 $\pm$ 5.7	23.8 $\pm$ 1.8	1471.9 $\pm$ 1387.7	2575.8 $\pm$ 520.4	8,8
	M3	1.2 $\pm$ 2.0	22.0 $\pm$ 12.0	22.5 $\pm$ 2.1	490.63 $\pm$ 2775.4	2207 $\pm$ 1387.7	11,8
	M4	5.3 $\pm$ 0.3	35 $\pm$ 0.7	24 $\pm$ 1.4	4906.3 $\pm$ 0.00	3925.0 $\pm$ 346.9	16,5
Canino	M0	6.91 $\pm$ 3.2	32.8 $\pm$ 4.0	23.3 $\pm$ 10.3	7031.5 $\pm$ 2660.4	6690.5 $\pm$ 2122.1	0
	M1	11.65 $\pm$ 2.7	42.5 $\pm$ 6.3	35.6 $\pm$ 8.2	11052 $\pm$ 3133.7	12327.3 $\pm$ 5085.2	14,6
	M2	15.52 $\pm$ 4.4	39.5 $\pm$ 26.6a	38.5 $\pm$ 221.5	11618.8 $\pm$ 22327.0	11760.5 $\pm$ 23370.1	10,6
	M3	11.00 $\pm$ 3.6	40.5 $\pm$ 6.1	22.0 $\pm$ 10.7	10060.2 $\pm$ 2870.1	9210.0 $\pm$ 3703.3	20
	M4	10.98 $\pm$ 4.5	28.9 $\pm$ 5.5	33.7 $\pm$ 17.2	9351.7 $\pm$ 1891.9	11335.4 $\pm$ 4614.4	70.9
Moraiolo	M0	5.8 $\pm$ 1.2b	28.4 $\pm$ 1.5	20.4 $\pm$ 6	5526 $\pm$ 1852.9c	5148.2 $\pm$ 572.6b	0
	M1	14.3 $\pm$ 4.9	36.3 $\pm$ 3.5	42.3 $\pm$ 10.6	15491.7 $\pm$ 6445.6	18892.3 $\pm$ 6926.0	13,8
	M2	17.3 $\pm$ 4.8	32.2 $\pm$ 3.5	38.7 $\pm$ 17	15491.7 $\pm$ 2852.7	15491.7 $\pm$ 5111.4	10,9
	M3	15.8 $\pm$ 2.4	38.0 $\pm$ 4	40.7 $\pm$ 10.6	12846.8 $\pm$ 1308.9	15869.6 $\pm$ 2267.1	16,1
	M4	16.1 $\pm$ 1.8	31.3 $\pm$ 4.2	45.0 $\pm$ 1	19648.0 $\pm$ 4719.3	18514.5 $\pm$ 2359.7	22,1

The leaf area was always superior in leaves collected from treated plants with AMF. However, the difference varied according the type of AMF (Table 3.8). This increase is probably due to the improvement of the mineral nutritional status, mainly phosphorus, as reported by Nagarathna *et al.* (2007) in sunflower leaves, by Wu and Xia (2006) in trifoliate orange and by Johnson (1982) in citrus.

Table 3.8: Mean leaf area of plants treated with arbuscular mycorrhiza

Cultivar	AMF	leaf area (mm ²)
Canino	M0	147,03 ^b
	M1	167,98 ^b
	M2	234,76 ^a
	M3	223,42 ^a
	M4	253,49 ^a
Moraiolo	M0	187,51 ^d
	M1	248,67 ^b
	M2	233,86 ^{bc}
	M3	207,44 ^c
	M4	270,11 ^a
Canino Eco15-5	M1	75,19 ^b
	M3	100,79 ^a
	M4	98,36 ^a
Canino AT17-2	M1	156,88 ^{ns}
	M2	167,31 ^{ns}
	M3	125,98 ^{ns}
	M4	156,88 ^{ns}
Canino AT17-3	M0	114,47 ^b
	M1	187,56 ^a
	M2	134,02 ^b
	M3	139,61 ^b
	M4	138,11 ^b
Canino AT17-10	M1	160,81 ^{ns}
	M4	142,53 ^{ns}

Note: Different letter for each group of cultivar indicate statistically different values per P=0.05

The total chlorophyll content (Table 3.9) was generally higher in treated plants with mycorrhiza, although not all the results resulted statistically significantly, in leaves collected from plants treated with AMF. Some variations were registered between in chlorophyll type (A and B) content in cultivars and type of AMF. Our data confirmed that AMF improved the nutrition processes and support higher chlorophyll content, similar like reported by (Paradis 1995).

Table 3.9: Mean of chlorophyll A, chlorophyll B and total chlorophyll content in leaves of olive plant after application with arbuscular mycorrhiza.

Cultivars	AMF	Chlorophyll A (mg/g fresh weight)	Chlorophyll B (mg/g fresh weight)	Total Chlorophyll (mg/g fresh weight)
Canino	M0	1,15 ^{ns}	1,51 ^b	2,61 ^d
Canino	M1	2,19 ^{ns}	3,38 ^a	5,57 ^a
Canino	M2	2,76 ^{ns}	1,55 ^b	4,32 ^b
Canino	M3	1,64 ^{ns}	1,86 ^b	3,50 ^c
Canino	M4	1,79 ^{ns}	2,77 ^a	4,56 ^{ab}
Moraiolo	M0	1,43 ^c	1,85 ^c	3,27 ^d
Moraiolo	M1	2,64 ^{ab}	4,83 ^a	7,47 ^a
Moraiolo	M2	2,74 ^a	3,50 ^{ab}	6,24 ^b
Moraiolo	M3	2,80 ^a	3,56 ^{ab}	6,36 ^b
Moraiolo	M4	2,40 ^b	3,12 ^{ab}	5,52 ^c
Canino Eco15-5	M2	1,19 ^{ns}	3,57 ^{ns}	4,76 ^{ns}
Canino Eco15	M3	1,35 ^{ns}	1,95 ^{ns}	3,30 ^{ns}
Canino Eco15	M4	1,38 ^{ns}	2,03 ^{ns}	3,41 ^{ns}
Canino AT17-2	M1	1,75 ^{ns}	2,34 ^{ns}	4,09 ^{ns}
Canino AT17-2	M2	1,49 ^{ns}	2,17 ^{ns}	3,66 ^{ns}
Canino AT17-2	M3	1,36 ^{ns}	1,94 ^{ns}	3,30 ^{ns}
Canino AT17-2	M4	1,06 ^{ns}	2,93 ^{ns}	4,74 ^{ns}
Canino AT17-3	M1	1,48 ^b	2,27 ^a	3,76 ^b
Canino AT17-3	M2	2,05 ^a	2,07 ^a	4,12 ^a
Canino AT17-3	M3	1,38 ^b	1,52 ^b	2,90 ^c
Canino AT17-3	M4	1,07 ^c	1,32 ^c	2,40 ^d
Canino AT17-10	M1	1,26 ^{ns}	1,97 ^{ns}	3,23 ^{ns}
Canino AT17-10	M4	1,22 ^{ns}	2,03 ^{ns}	3,25 ^{ns}

Note: Different letter for each group of cultivar indicate statistically different values per P=0.05

IV. CONCLUSIONS

This study demonstrated that AMF, developed in different areas, could be beneficial also for other native olive plants. Although it has been tried to obtain AM by *in vitro* technique by using transgenic roots, it was not possible due to the high level of microbial contamination. Therefore the traditional methods by using sorghum as host plant with pot culture technique has been used with success. In addition, the AMF were analysed through the investigation of morphological and physiological characters, as well as molecular analysis. The molecular study did not allow us to distinguish between *Gigaspor. albida* and *G. rosea*, while morphological analysis allowed us to separated between them.

It has also been demonstrated that transgenic olive plants used for experiments (osmotin and *rolABC* Canino) maintained the transgenic gene after several years of cultivation *in vitro*. This is essential for demonstrating the validity of genetic transformation for the improvement of the transgenic olive plant *in vitro*.

Olive micropropagation used for the experiment has also been improved, both in proliferation and rooting. Dikegulac increased shoot proliferation of olive by decreasing apical dominance. The study on light photoperiod has been conducted of both olive cultivars, and it gave important information about the light culture condition for both *wild type* and transgenic Canino cultivar to increase rooting ability. Four days in the dark and then in the light until the end of rooting phase was the best conditions which allowed high rooting ability of olive shoots

The positive effect of AMF native from Indonesia on the survival rate and growth of olive plant in *post vitro* has been observed. The results showed that responsiveness degrees of AMF inoculants not only vary according to olive cultivar but also on the type of AMF. Inoculated non transgenic plants showed higher response to AMF in root colonization than both transgenic olive plants (osmotin and *rolABC*). The plant growth achieved through the application of AMF might was due to their capability of increasing plant nutrient uptake. Inoculation with AMF appears to become essential for the survival and growth of micropropagated olive plants.

Due to the results of AMF to micropropagated olive plants it is necessary to develop the method with others factors such as media composition, nutrition, and

inoculums quality of AMF and as well with a more appropriate substrate and technique of cultivation, higher number of plants and to be parameters observe. It is also important to study the effect of AMF native to Italy area and compare it with its development in different areas.

The results obtained in this study showed the importance of exploring and exploiting the natural diversity of AMF as a starting point to formulate inoculants to be applied as inoculums to in an attempt to plant growth.

V. ATTACHMENT

Attachment 1. FASTA format of SSU sequence

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Attachment 2. Fasta format of ITS sequence

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VII. ACKNOWLEDGMENTS

This thesis dissertation has been carried out at the *Dipartimento di Produzione Vegetale* at *Universita degli Studi della Tuscia*, Viterbo – Italy, and this PhD was one of the most important experience in my life for which there are many people that I would like to acknowledge for their help, support, and guidance. I could not have done what I was able to do without them.

First of all, I wish to express my big gratitude to my tutor Prof. Eddo Rugini, for his continued encouragement, patience and invaluable suggestions during this work and careful reading of all of my writing.

I would like to thanks to Prof. Manuela Giovannetti from Pisa University, who gave me opportunity to study and working in her laboratory with her great researcher team Dr Alessandra Turini, .Prof. Luciano Avio, and Dr. Cristina Sbrana. Thank you so much for theirs scientific advice and knowledge on arbuscular mycorrhiza. I feel so honourable that I can know them and working with them.

I'm also very grateful to Prof. Rosario Muleo who has offered me an opportunity to study and extend my research on molecular study, thank you very much for his guidance and helpful discussions.

I would also like to thank Prof. Francesco Saccardo and Prof. Albero Graifenberg, for their helpful career advice and suggestions in general.

I will forever be thankful to Dr. Patricia Gutierrez Pesce, Dr. Emilio Mendoza and Dr. Chiara Colao for their friendship and support provided, they have been helpful in providing advice, encouragement and editing assistance for many times during my PhD course. Thanks a lot to Dario and Roberta Lupi, Tziziana and Massimo, for help me in my first year in the Tree Eco-physiology laboratory. I also thank the staff of plants biotechnologies and micropropagation laboratory Mara, Antonella, Corado and Claudio. Thanks to Dr. D. Torino, Dr G. Ubertini and Dr. S. Marinari for their help during my working here.

A particular thank to my friends colleagues and Dr Yenni Bakhtiar, Prof, Nadirman Haska, my supervisor and head of main office in The Center of Application of

Biotechnology, The Agency of the Assessment and Application of Technology – Indonesia, for giving me continued permission and encouragement during the work.

I would like to thank also to all my friends in Italy for the nice moments we spend together and their help, Gina, Mauro, Pili, Claudia, Marwan, Giorgia, Ljiljana Kuzmanovic, Lina Maria, Marco, my friends from DABAC and all my friends in the university, Thank you very much!!!

Big Thanks!! to my PhD colleagues particularly Chiara Lo Bianco, Maria Elena Provenzano, Deborah Pagliacca, Leonardo Parrano, Enrico Scarici, Carlo Falovo Barbara, Alessia, Deborah, Marco and Roberto Ruggeri thank you a lot for the support and the time we spend together during this years.

A special thanks to my “concolina” Katia Liburdi I’ll be miss you, whenever I met difficulties, you are always standing beside me and give me a hand. Terima kasih katia

In the end I recognize that this experience would not have been possible without the financial support from the Ministry Foreign Affair Italy, The Italian Institute of Culture Jakarta and express my gratitude to those agencies.

Finally, certainly not the last, a special thought is devoted to my parents and my family for the support and love they provided me through my entire life. Love you all. The encouragement and support from my beloved Mum and Dad is a powerful source of inspiration and energy.

Save...farida..save!!

Alhamdullilah Terima kasih Grazie per tutto..... a tutti