DEPARTMENT OF AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES

DEGREE COURSE: MASTER COURSE IN AGRICULTURAL SCIENCE

DEVELOPMENT OF IN VITRO STERILIZATION AND PROLIFERATION PROTOCOLS OF LOCAL OLIVE VARIETY

(MESSO A PUNTO DI PROTOCOLLI DI STERILIZAZIONE E PROLOFERAZIONE IN VITRO DI VARIETÀ LOCALI DI OLIVO)

TYPE OF DISSERTATION: Experimental

Student:

ZAHRA MOHAMMADI

Supervisor:

PROF. BRUNO MEZZETTI

Assistant supervisor

DR. LUCA CAPRIOTI

DR. SILIVIA SABBADINI

ACADEMIC YEAR 2022-2023

Abstract (English)

Micropropagation is a pivotal clonal propagation technique that utilizes tissue culture, with the ability to overcome challenges associated with traditional methods. It offers efficient and uniform plant production, with advantages including precise control of overgrowth conditions, rapid multiplication, and the ability to assess the genetic and sanitary health of micro-propagated plants (Hassan and Zayed, 2018). However, there are issues in this procedure, such as tissue oxidation and challenges in obtaining sterile plant material and establishing sterile shoot cultures. Therefore, the development of *in vitro* micropropagation protocols is essential. This study, conducted at the Plant Biotechnology Laboratory of Marche Polytechnic University, focused on local olive varieties, including 'Ascolana Tenera,' 'Piantone di Mogliano,' 'Piantone di Falerone,' and 'Maurino.' The aim was to improve *in-vitro* techniques for sterilization by testing various concentrations and exposure times of sodium hypochlorite and hydrogen peroxide and optimize proliferation by examining eight proliferation media to select the best plant growth regulator (PGR) types and concentrations for enhancing shoot proliferation. The results indicated that sodium hypochlorite, especially treatments 5 (15% for 15 minutes) and 6 (15% for 10 minutes), was more effective than hydrogen peroxide. 'Maurino' demonstrated higher resistance to higher sodium hypochlorite concentration. In the proliferation phase, distinct trends were observed among different PGR combinations. Proliferation medium 4 (3 mg/L 6-Benzyl-aminopurine (BAP) and 0.5 mg/L Zeatin) efficiently supported healthy explant development, while medium 8 (5 mg/L BAP) consistently outperformed others in promoting shoot development. Despite some susceptibility to necrosis, the positive effects of Zeatin and BAP on olive explant cultivation were consistent with other research.

This study provides valuable insights into developing efficient micropropagation protocols for local olive cultivars, contributing to the advancement of *in-vitro* cultivation techniques for this economically important species. Future research could further refine these protocols, considering the specific needs of different olive cultivars.

Abstract (Italian)

La micropropagazione è una tecnica cruciale di propagazione clonale che utilizza la coltura di tessuti, con la capacità di superare le sfide associate ai metodi di propagazione tradizionali. Tale tecnica offre una produzione efficiente e uniforme di piante, con vantaggi che includono il controllo preciso delle condizioni di crescita, la rapida moltiplicazione e la capacità di valutare la componente genetica e lo stato sanitario delle piante micropropagate (Hassan and Zayed, 2018). Tuttavia, vi sono problemi in questa procedura, come l'ossidazione dei tessuti e le sfide nell'ottenere materiale vegetale sterile e nell'istituire colture di germogli. Pertanto, lo sviluppo di protocolli di micropropagazione *in vitro* è essenziale. Questo studio, condotto presso il Laboratorio di Biotecnologia Vegetale dell"Università Politecnica delle Marche, si è concentrato su varietà locali di olivo, tra cui 'Ascolana Tenera,' 'Piantone di Mogliano,' 'Piantone di Falerone,' e 'Maurino.' L'obiettivo è stato quello di adattare la sterilizzazione e la stabilizzazione alla coltura *in vitro* di espianti di olivo, testando varie concentrazioni e tempi di esposizione con ipoclorito di sodio e perossido di idrogeno, e l'ottimizzare la proliferazione esaminando otto substrati di proliferazione per selezionare i migliori tipi e concentrazioni di regolatori della crescita delle piante (PGR) per potenziare la proliferazione dei germogli. I risultati indicano che l'ipoclorito di sodio, in particolare i trattamenti 5 (15% per 15 minuti) e 6 (15% per 10 minuti), è più efficace del perossido di idrogeno. 'Maurino' ha dimostrato una maggiore resistenza a concentrazioni più elevate di ipoclorito di sodio. Nella fase di proliferazione, sono emerse tendenze distinte tra diverse combinazioni di PGR. Il substrato di proliferazione 4 (3 mg/L di 6-Benzilaminopurina (BAP) e 0.5 mg/L di Zeatina) ha supportato efficientemente lo sviluppo di germogli sani, mentre il substrato 8 (5 mg/L di BAP) è risultato il più efficiente nel promuovere lo sviluppo dei germogli. Nonostante una certa suscettibilità alla necrosi, gli effetti positivi di Zeatina e BAP sulla coltivazione e moltiplicazione degli espianti di olivo sono stati coerenti con altre ricerche.

Questo studio fornisce preziosi spunti nello sviluppo di protocolli efficienti di micropropagazione per le varietà locali di olivo, contribuendo all'avanzamento delle tecniche di coltivazione *in vitro* per questa specie economicamente importante. Ricerche future potrebbero perfezionare ulteriormente questi protocolli, considerando le esigenze specifiche delle diverse varietà di olivo.

Acknowledgements

I would like to thank the **Marche Polytechnic University** for affording me the valuable opportunity to pursue my master's course here.

In addition, I would like to express my sincere gratitude to my supervisor, **Prof. Bruno Mezzetti**, for the continuous support of my thesis and immense knowledge.

My appreciation also extends to my co-supervisor, **Dr. Luca Capriotti**, for his patience, mentoring with a smile, and encouragement.

Also, I want to express my thanks to my other co-supervisor, **Dr. Silvia Sabbadini**, for her kind advice and guidance during this academic journey.

I would like to thank all **colleagues at the Plant Biotechnology Laboratory of the Polytechnic University of Marche**.

Last but not least, I want to thank all **professors**, **friends**, and **tutors** who kindly supported me during this master's course.

Dedication

This dissertation is dedicated to my husband Arash and daughter Luna whose love, support, encouragement and patience have enriched my soul and inspired me to pursue and complete this research

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACRONYMS AND ABBREVIATIONS

ROM	Ruggini Olive Medium
PGR	PLANT GROWTH REGULATOR
BAP	6-benzylaminopyrine
IOC	International Olive Council
SE	STANDARD ERORR
NaClO	Sodium hypochlorite
H2O2	Hydrogen peroxide
P1	Proliferation medium 1
P2	Proliferation medium 2
P3	Proliferation medium 3
P4	Proliferation medium 4
P5	Proliferation medium 5
P6	Proliferation medium 6
P7	Proliferation medium 7
P8	Proliferation medium 8

CHAPTER 1
INTRODUCTION

1.1) Olea europaea

Olive (*Olea europaea* L.), a member of the Oleaceae family (Table 1), is an evergreen tree or shrub that was originally domesticated in the fertile crescent region, from where it spread later on, to many parts of the world with a Mediterranean climate (Fraga *et al*., 2019; Khosravi *et al*., 2021). However, currently, the expansion of olive cultivation has extended to numerous non-traditional producer countries, including Argentina, Australia, Chile, China, and the United States (Diez *et al*., 2015). The cultivation of olive trees has mainly been practiced for fruit production, the extraction of oil, and various other products. While the oil obtained from the fruit mesocarp serves as a valuable and nutritious food source, its significance in antiquity extended to diverse applications such as lamp fuel, wool treatment, medicinal and cosmetic purposes, as well as soap production, among others (Therios, 2009). Archaeological excavations have revealed olive remains dating back to the Palaeolithic era in Greece (around 60–50,000 years ago) (Friedrich,1980; Fraga *et al*., 2019). The earliest indications of olive cultivation date back to 4 B.C. century and even earlier (Zohary and Spiegel-Roy 1975).

Table 1: Scientific classification of olive (Modified from Therios, 2009)

Kingdom	Green Plants
Subkingdom	Tracheobionata - vascular plants
Super division	Spermatophyta - seed plants
Division	Magnoliophyta - flowering plants
Class	Magnoliopsida - Dicotyledons
Sub Class	Asteridae
Order	Scrophulariales or Lamiales
Family	Oleaceae - ash, privet, lilac, and olives
Genus	*Olea*
Species	*europaea*

The olive tree can be successfully cultivated across a range of climatic and soil conditions, encompassing the entire temperate and subtropical zone, specifically between 30° and 45° (Palliotti and Bongi, 1996). Regions dedicated to olive cultivation typically experience a mean annual temperature of 15–20°C, with a minimum of 4°C and a maximum of 40°C. To avoid damage to the trees, the minimum temperature must not fall below –7°C. However, this threshold is approximate, as the resilience of olive trees is influenced by varied factors such as the duration of low temperatures, atmospheric humidity, and the specific cultivar. Damage may also vary between individual trees. In cases of gradual temperature decline, olive trees can endure temperatures as low as –12°C (Palliotti and Bongi, 1996; Therios, 2009).

1.2) Morphology of the olive

The olive trees are characterized by their extraordinary longevity, therefore, in suitable environments finding millenary olive trees is common.

1.2.1 Root system

In the olive tree, the majority of roots tend to thrive at a soil depth ranging from 60 to 70 cm, with thicker roots predominantly developing within the top 20 cm. In arid regions, the root system extends over an area seven to eight times larger than the leaf area (Fernández *et al*., 1990). In regions with dense soil, fibrous roots are found near the soil surface, while in sandy or light-textured soils, the root system tends to be expansive. Therios (2009) explained the lateral growth of roots spans 12 meters from the trunk and reaches a depth of 6 meters. This deep-rooted system enables the olive tree to extract water and nutrients from soils with minimal rainfall (Therios, 2009). The functionalities of the olive root system are described as 1) anchorage of the tree. 2) water and nutrient absorption. 3) synthesis of various organic materials. 4) storage of nutrients. The role of the root system is dependent on factors such as the rootstock, variety, soil conditions, and cultivation practices (Martínez-Ballesta *et al*., 2010).

1.2.2 Trunk

The olive trunk takes on a cylindrical shape with an irregular surface marked by numerous swellings. The wood exhibits a yellowish hue that deepens towards the center of the trunk (Therios, 2009). The height of the generation of branches on the trunk and the number of them is determined by the grove system. The primary branches extend into secondary and tertiary branches, which bear the leaves, flowers, and fruits. The small shoots are classified into four categories of vegetative, fruit-bearing, mixed, and water sprout (sucker).

1.2.3 leaves

The leaves of the olive tree are simple, elliptical to lanceolate in form, and have smooth margins (Moreno-Alías *et al*., 2009). The foliage typically persists on the tree for 2 to 3 years, though there can be variations. The upper surface of the leaf is characterized by a dark green hue, while the underside is adorned with white

flakes, displaying a color spectrum ranging from white to silvery. The leaf sheath measures between 3 to 8 cm in length and varies in width from 1 to 1.8 cm (Figure 1) (Fabbri *et al*., 2009).

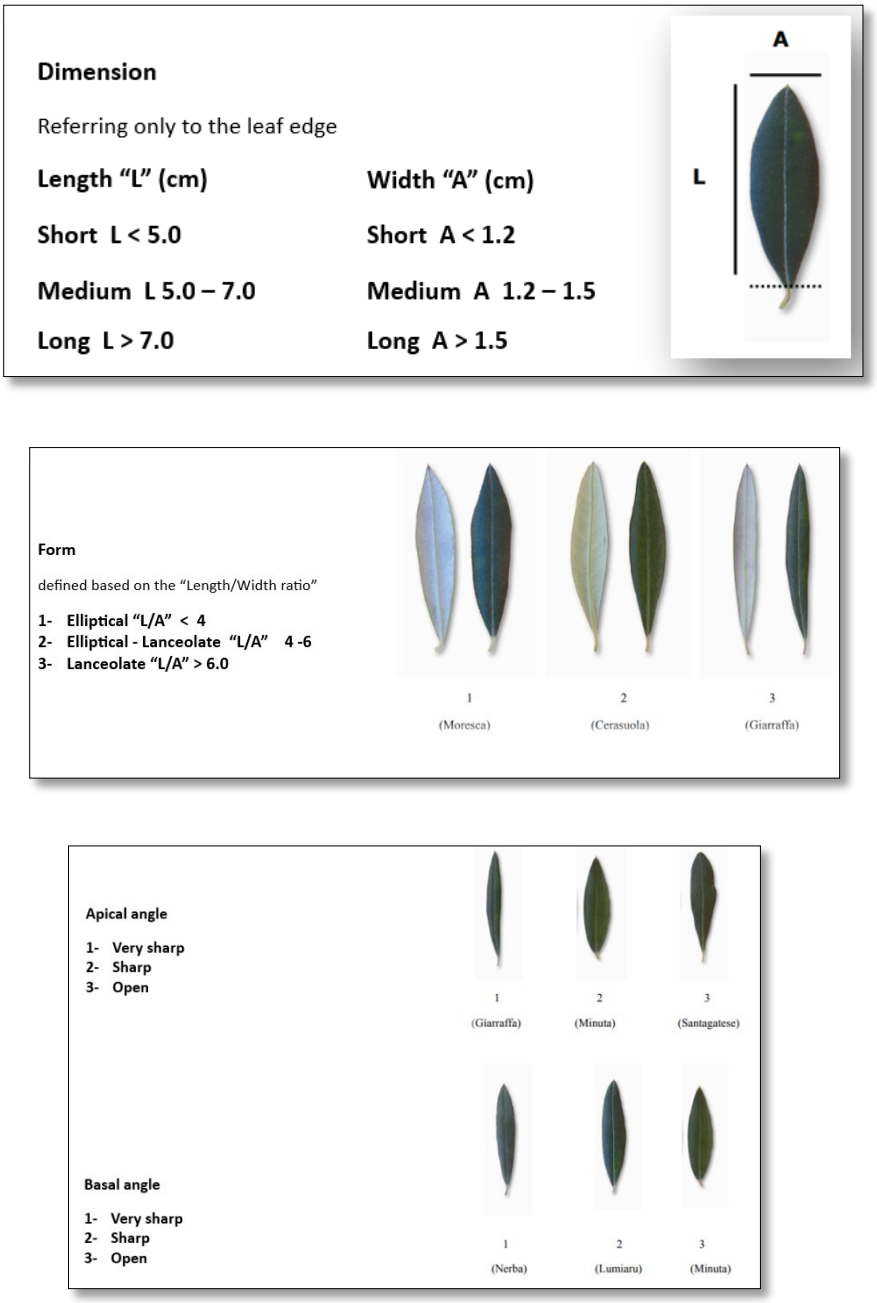

Figure 1: Characteristics of olive leaves (Modified from DE SIMONE and CARUSO, 2014).

However, there are several morphological differences between adult and juvenile leaves including leaf size and weight, thorniness, pigment accumulation, and phyllotaxy (Hackett, 2011; Moreno-Alías *et al*., 2009).

1.2.4 Inflorescences and flowers

Inflorescences arise at the junction of each leaf (leaf axil) (Figure 2) (Therios, 2009). Each of these inflorescences contains several flowers. flower buds are formed on the developing shoots the year before anthesis (Fabbri *et al*., 2009). Two types of flowers are present each season: “perfect flowers” (Figure 3), containing stamens and pistils, and “staminate flowers”, containing aborted pistils and functional stamens. “Ovary abortion” refers to the absence of an ovary or a small, imperfect, non-persistent ovary (Fabbri *et al*., 2009).

Figure 2: Olive inflorescence before blooming (Top) and after blooming (down) Modified from Milicevic *et al*., 2020)

Figure 3: Perfect and imperfect flowers. The flowers in top are complete; The down ones have been cut in half to be able to better visualize the structure of the pistil Olive flowers (Adapted from Barranco *et al*., 2008)

1.2.5 Fruit

The olive fruit is a drupe, taking on a spherical or elliptical shape, and is composed of three layers: the epicarp (skin) containing stomata, the mesocarp (flesh) representing the edible part, and the endocarp (pit) housing the seed (Therios, 2009). Fruit shape and size, as well as variations in pit size and surface morphology, display significant diversity among cultivars and are reliable morphological traits to distinguish between cultivars (Fabbri *et al*., 2009). Olive fruit growth can be described by a double-sigmoid growth curve (Fernández *et al*., 2018; Gucci *et al*., 2008), which is divided into four growth phases. The first phase (I Phase) is characterized by rapid cell division, which induces an exponential expansion of the volume; in the second phase (II Phase) the speed of cell division diminishes, while the pit is hardening and fruit size increases slowly; in the third phase (III Phase) the fruit dimension increases with rapid and linear growth continues and leads to a beginning of fruit maturation; the fourth phase (IV Phase) finishes with full ripening and eventually fruit drop (Khosravi *et al*., 2021).

1.3 Olive fruit and oil components

As mentioned previously, in a botanical point of view, olive fruit can be divided into three parts: epicarp, mesocarp, and endocarp. The epicarp, constituting 1.0–3.0% of the drupe weight, included a waxy layer that covers the skin, making up 45–70% of its weight. In the initial stages of development, the skin appears green due to chlorophyll, transitioning to straw yellow, pink, purple, and ultimately black as it matures (Therios, 2009).

The mesocarp, constituting 70–80% of the entire olive fruit, holds paramount significance as it is the edible portion. The flesh has a water content ranging from 70 to 75% of its weight, while the oil content varies between 14 and 15% in green table olives and can reach up to 30% in mature black olives. Olives contain oxalic, succinic, malic, and citric acids and prominent levels of free fatty acids. The sugars are mainly represented by glucose, fructose, saccharose, and mannitol (Therios, 2009).

The endocarp constitutes 10–27% of the olive's weight, with the seed making up 2–4% of this total. The seed contains 22–27% oil, while the shell accounts for 1%. Key characteristics of the endocarp include size, weight, and ease of separation to the flash (Therios, 2009).

Olive oil primarily consists of triacylglycerols, constituting approximately 99% of its composition. Additionally, it contains secondary components such as free fatty acids, mono- and diacylglycerols, as well as various lipids including hydrocarbons, sterols, aliphatic alcohols, tocopherols, and pigments (Table 2). Furthermore, olive oil contains a diverse range of phenolic and volatile compounds (Boskou *et al*., 2006).

Five main classes of phenolic compounds have been identified in olive fruit, olive oil, and in milling by-products: secoiridoids and their derivatives, phenylpropanoids, flavonoids, simple phenols, and lignans (Cecchi *et al*., 2013).

The phenol content in olive oil can differ from one variety to another, and it is influenced by factors such as climate, harvesting and production techniques, the level of olive maturity, and preservation methods (IOC, http://www.internationaloliveoil.org/). In addition to polyphenols, olive oil contains large quantities of vitamins A, D, and K, along with vitamin E (IOC).

Table 2: Fatty Acid Composition as Determined by Gas Chromatography (% m/m methyl esters) (Adopted from Boskou *et al*., 2006).

Fatty Acid		Codex Alimentarius (2003)	IOOC*(2003)
lauric	C12:0	Not present in discernible amounts	Not specified
myristic	C14:0	< 0.1	< 0.05
palmitic	C16:0	7.5-20.0	7.5-20.0
palmitoleic	C16:1	0.3-3.5	0.3-3.5
heptadecanoic	C17:0	< 0.5	≤ 0.3
heptadecenoic	C17:1	< 0.6	≤ 0.3
stearic	C18:0	0.5-5.0	0.5-5.0
oleic	C18:1	55.0-83.0	55.0-83.0
linoleic	C18:2	3.5-21.0	3.5-21.0
linolenic	C18:3	**	≤ 1.0
arachidic	C20:0	0.8	≤ 0.6
eicosenoic	C20:1	Not specified	≤ 0.4
behenic	C22:0	< 0.3	≤ 0.2***
erucic	C22:1	Not present in discernible amounts	
lignoceric	C24:0	< 1.0	≤ 0.2

*The limits established include the precision values of the recommended method; **pending the results of IOOC survey and further consideration by the Committee on Fats and Oils, national limits may remain in place; ***Limit raised to <0.3 for olive-pomace oils.

1.4 Main olive oil producers

The olive tree holds a paramount economic significance as the primary oil-producing crop in numerous Mediterranean countries. Presently, approximately 90% of olive trees are cultivate d in these regions, notably in Spain, Italy, and Greece. Furthermore, olive tree cultivation has extended to include countries such as Australia, China, Latin America, South Africa, and the USA (Hatzopoulos *et al.,* 2002). As many as 1250 different cultivars of olive tree have been cultivated globally in 54 countries (Abuzayed *et al*., 2018). Olive oil production has tripled in the last 60 years to reach 3 266 500 t in the 2019/20 crop year., Spain, Italy, Greece, Tunisia, Turkey, and Morocco are the world's largest producers (Figure 4).

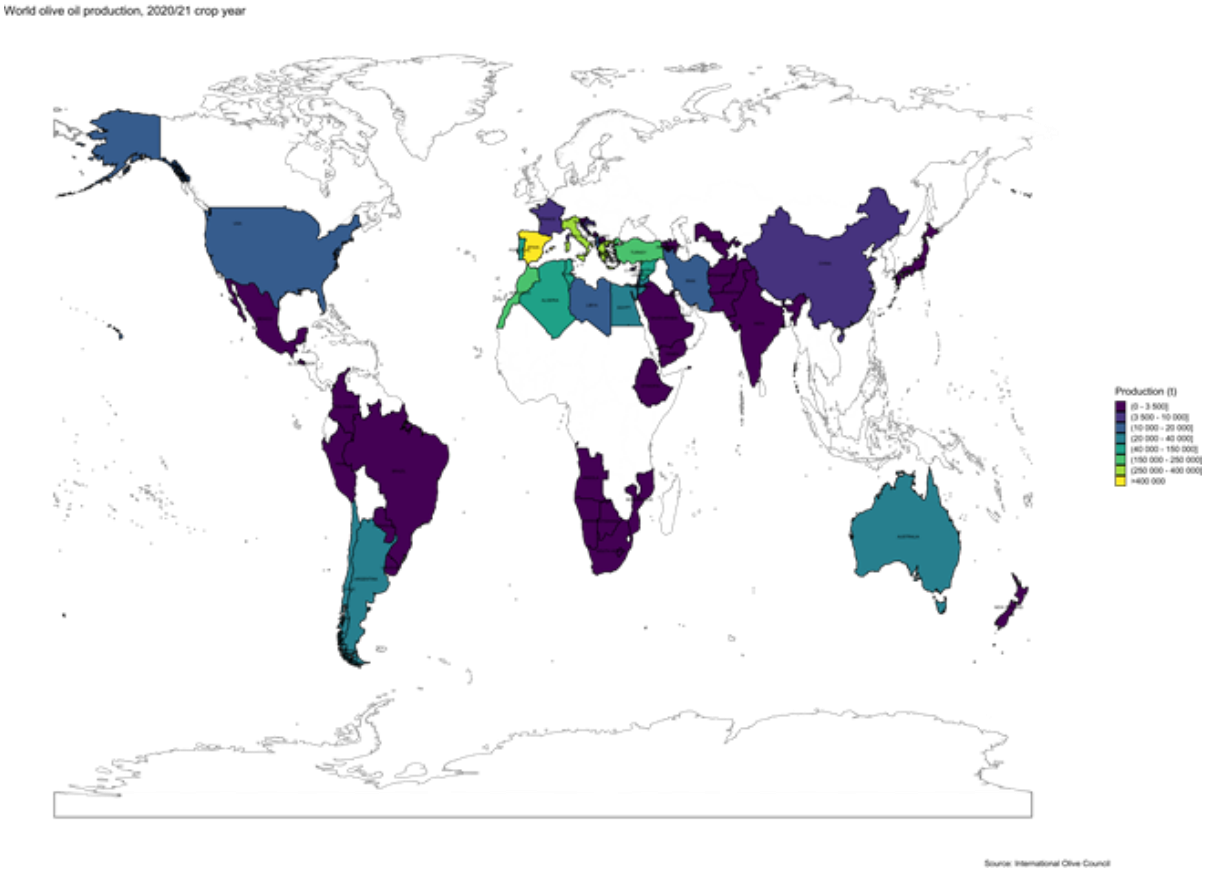

Figure 4: Map of Olive oil production 2020/21 (Source: www.internationaloliveoil.org)

The largest European producers of olive oil according to the International Olive Council are Spain, Greece, Italy, and Portugal. According to official country data and Executive Secretariat estimates, world production is forecast at around 3 098 500 t in the 2021/22 crop year, 2.9% up on the previous year (IOC) (Table 3). Figures 5 and 6 illustrate global and European Union olive oil production, respectively. Figure 5 provides insights into worldwide trends, while Figure 6 focuses specifically on olive oil production within the European Union.

Table 3: Olive oil production (Source: www.internationaloliveoil.org)

Production (×1000tn)	2016/2017	2017/2018	2018/2019	2019/2020	2020/2021(p.)	Average	2021/2022(e.)	%variation rates
EU, of which:	**1 752**	**2 188**	**2 264**	**1 920**	**2 051**	**2 035**	**1 974**	↓ -3.8%
SPAIN	1 291	1 262	1 790	1 125	1 389	1 371	1 300	↓ -6.4%
GREECE	195	346	185	275	275	255	225	↓ -18.2%
ITALY	182	429	174	366	274	285	315	↑ 15.2%
PORTUGAL	69	135	100	140	100	109	120	↑ 20.0%
Other IOC countries, of which:	**620**	**1 007**	**808**	**1 158**	**758**	**870**	**936**	↑ 23.5%
TUNISIA	100	325	140	440	140	229	240	↑ 71.4%
TURKEY	178	263	194	230	210	215	228	↑ 8.3%
MOROCCO	110	140	200	145	160	151	200	↑ 25.0%
ALGERIA	63	82	97	126	70	88	98	↑ 39.0%
EGYPT	30	40	41	40	30	36	20	↓ -33.3%
ARGENTINA	24	45	28	30	30	31	30	↓ 0.0%
Non-IOC producers:	**190**	**184**	**233**	**188**	**200**	**199**	**188**	↓ -6.2%
TOTAL	**2 561**	**3 379**	**3 305**	**3 266**	**3 010**	**3 104**	**3 099**	↑ 2.9%

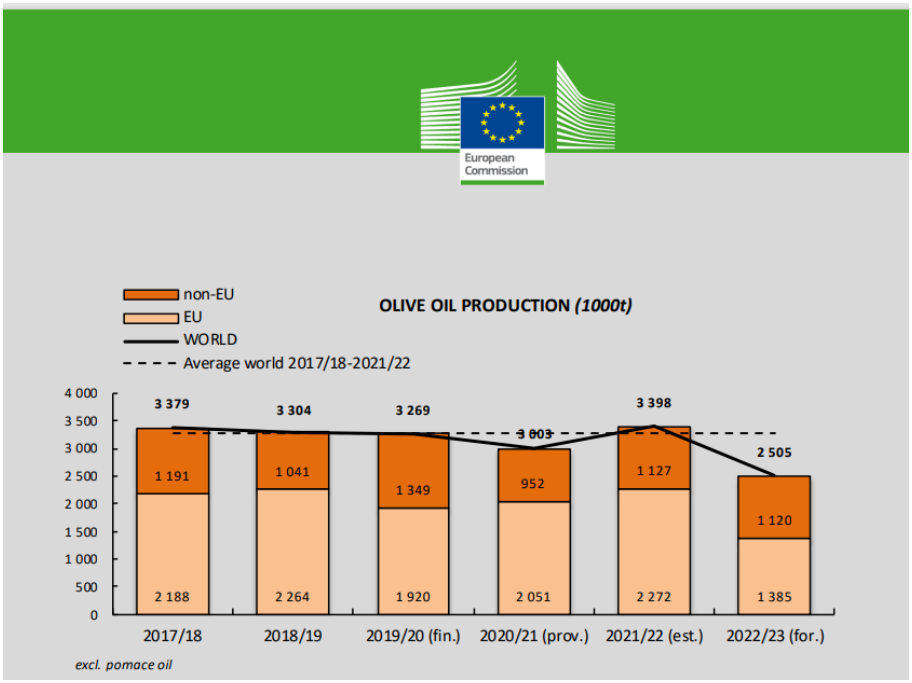

Figure 5: Olive oil production in the world (https://agriculture.ec.europa.eu/)

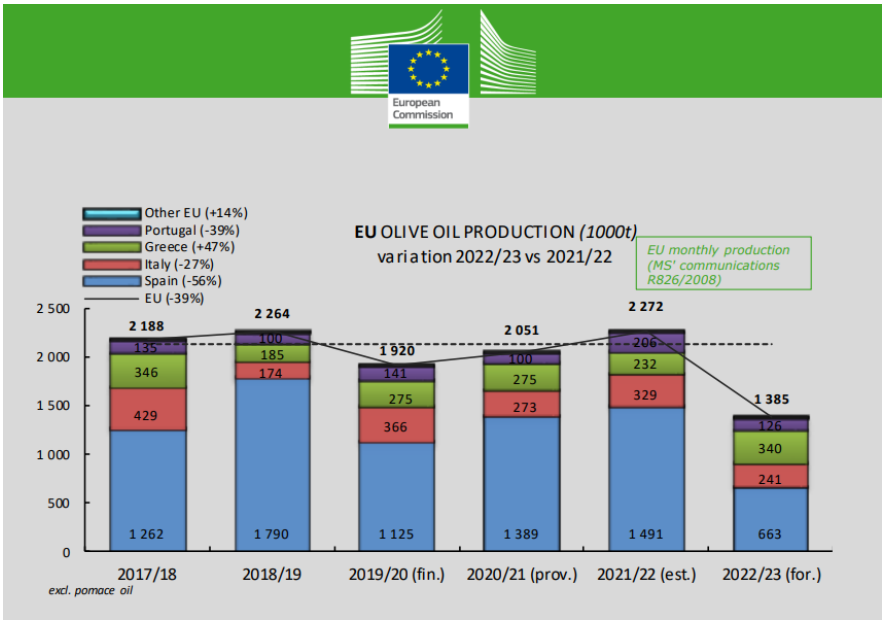

Figure 6: Olive oil production in the European Union (https://agriculture.ec.europa.eu/).

1.5 Main world cultivars

In the Mediterranean basin, where a majority of germplasm is concentrated, the colonization of numerous renowned varieties around the world has originated. The main important cultivars are shown in Table 4.

Table 4: Several important olive cultivars. Modified from (Fabbri *et al*., 2009).

Cultivar	Origin	Cultivar	Origin	Cultivar	Origin
Mansalina	Spain	Leccino	Italy	Ascolana tenera	Italy
Sevillana	Spain	Frantoio	Italy	Koroneiki	Greece
Arbequina	Spain	Pendolino	Italy	Amphisis	Greece
Arbosana	Spain	Coratina	Italy	Mastoidis	Greece
Picual	Spain	Moraiolo	Italy	Kalamata"	Greece
Hojiblanca	Spain	Biancolilla	Italy	Picholine	France
Cornicabra	Spain	Nocellara del Belice	Italy	Lucques	France

Spain, a leading producer of both table olives and olive oil, boasts a rich diversity of olive cultivars, totaling 183 (Bartolini *et al*., 1998). Notably, "Sevillana" and "Manzanilla" stand out as key table cultivars, with "Spanish Sevillana" extending its cultivation to Algeria. The global presence of "Manzanilla" is also noteworthy. Apart from table olives, Spain is home to several pivotal cultivars primarily cultivated for oil production. Among these, "Picual" commands nearly one-third of the country's planted surface, making it one of the most widely grown varieties globally. "Arbequina" holds significance not only in Catalonia, Spain, but has found extensive use in new plantations in Argentina and Chile, gaining prominence due to its suitability for mechanical harvesting. Additionally, "Hojiblanca," predominantly cultivated in the Cordoba district of Andalusia, yields oil of good quality (17%) and can double as a table olive (Barranco, 1999). Other noteworthy oil cultivars include "Cornicabra" and "Morisca".

The distinctive nature of Italian olive culture stems from the extensive array of cultivars, is a consequence of the early introduction of the species, diverse environmental conditions within the country, and historical political fragmentation. With as many as 538 cultivars and over 1,300 synonyms reported (Bartolini *et al*., 1998), "Frantoio" emerges as a significant oil cultivar, originating in Tuscany but now widely distributed across different regions under various names (synonyms). Its adaptability to diverse climates has led to its adoption in emerging olive-growing countries like the United States, Australia, South Africa, Argentina, and Chile, owing to both its adaptability and the high quality of its oil. "Leccino", another pivotal Italian oil cultivar, hails from central Italy and exhibits notable resistance to harsh frosts, making it a focus in breeding programs aimed at selecting cold-resistant olive genotypes (Fabbri *et al.,* 2009). "Pendolino" finds extensive use in central Italian olive orchards, valued for its role as a pollinator. Additional noteworthy Italian oil cultivars include "Coratina," "Canino," "Carolea," "Moraiolo," and "Biancolilla". While Italy holds a prominent position in olive oil production, it does not feature among the leading countries for table olive production. Nonetheless, it boasts large-fruited cultivars that yield high-quality table olives. The "Nocellara

del Belice," akin to the Spanish "Manzanilla" and the Greek "Amphisis," stands out as the premier Italian table olive cultivar. "Ascolana tenera" is another vital Italian cultivar, now dispersed in countries such as Israel, Mexico, Argentina, and California (Fabbri *et al*., 2009). Noteworthy, dual-purpose cultivars including "Itrana," "Giarraffa," and "Tonda Iblea" are good alternative for producing high quality fresh product and olive oil. In Greece, the primary oil variety is the "Koroneiki," covering over 50% of the country's olive area, particularly in the Peloponnesus, Crete, and other islands. "Mastoidis", "Kalamata" ("Kalamon"), and "Chalkidiki" stand out as significant Greek varieties, characterized by their high oil content and favorable flesh-to-stone ratio. The primary black table olive cultivar in Greece is "Amphisis" ("Konservolia"), contributing to 80–85% of the country's table olive production, particularly thriving in central Greece. Picholine," cultivated in Southern France, is a prominent dual-purpose cultivar alongside "Lucques," playing a central role in the French table olive industry. This variety is also grown in Italy, Israel, Morocco, and occasionally in other olive-growing countries (Fabbri *et al*., 2009).

1.6 Main olive cultivars of Marche region

In recent times, numerous initiatives have been undertaken at the provincial level to preserve historic olive trees. These trees serve as witnesses to the olive tree's enduring presence in the region over centuries, symbolizing a profound connection with the local landscape and environment. They are authentic symbols of environmental harmony. One such initiative involves the characterization of native olive tree varieties in the Marche region (Figure 7). Additionally, a list highlighting the most significant olive varieties in the Marche region has been published as part of these preservation efforts (Figure 7) (https://www.assam.marche.it).

Figure 7 shows the distribution and the list of local olive cultivars in the Marche region.

Figure 7: Distribution of Local olive cultivar of Marche region (Source: https://www.assam.marche.it)

1.6.1 Olive cultivar "Ascolana Tenera"

This cultivar is native to the province of Ascoli Piceno but is occasionally cultivated throughout the entire Marche region (Figure 8) (www.assam.marche.it). The leaves are elliptical, with a thick, wavy, and helical lamina, a large surface area, and a deep green color on the upper side. Both basal and apical angles are open. The length of the leaf is 6.41 ± 0.81 cm (mean ± standard error), and its width is 1.54 ± 0.03 cm (mean ± SE). The tree produces medium-length inflorescences with a compact structure. The fruits have an ovoid shape and are slightly asymmetric. Drupes have a considerable size (4-8 g), and are subjected to weight changes with the load, and characterized by an intermediate oil yield. The fruit has very high pulp-to-pit ratio and late and gradual veraison; the color of the fruit changes from green to straw yellow until the time of harvest, resembling a table olive until ripening to a ripe purple (Figure 8). The pulp has a low consistency from the early stages of maturation. The tree exhibits high vigor, a voluminous canopy with high density, and fruit-bearing twigs that are medium-pendulous and branched. The optimal harvest period is around the end of September to the beginning of October for table olives and by the month of November for oil extraction. (www.assam.marche.it).

Figure 8: Olive cultivar Ascolana Tenera (Source: https://www.assam.marche.it)

1.6.2 Olive cultivar "Piantone di Falerone"

This cultivar, widely found in the province of Fermo, particularly in the area between the municipalities of Falerone and Montegiorgio, as well as in the inland areas of the province of Macerata, extending to high altitudes (Figure 9) (www.assam.marche.it). The leaves are elliptical- lanceolate, they are narrow and elongated, with a dark green color on the upper side. Both basal and apical angles are very sharp. The length of the leaf is 5.06 ± 0.79 cm (mean ± standard error), and its width is 1.01 ± 0.02 cm (mean ± SE). The tree has inflorescences with considerable length. The fruits have an ovoid shape and are symmetric. Drupes are medium size (2-2.5 g), characterized by medium to high oil yield. It has high pulp-to-pit ratio and late and contemporary veraison; the color of the fruits changes from light green to purplish black yellow until the time of harvest. Pulp consistency and resistance to detachment are low from early stage of maturation and progressively decrease. The tree exhibits medium vigor, an abundant foliage characterized by high density. The optimal harvest period is by mid-November (www.assam.marche.it).

Figure 9: Olive cultivar Piantone Falerone (Source: https://www.assam.marche.it)

1.6.3 Olive cultivar "Piantone di Mogliano"

This cultivar originates from the province of Macerata, with a greater concentration in the municipalities of Mogliano, Macerata, and the surrounding areas. It extends into the inland areas of the province, reaching altitudes above 600 meters above sea level." (Figure 10) (www.assam.marche.it). The leaves are elliptical-lanceolate, they are narrow and elongated, with a dark grayish-green color on the upper side. Both basal and apical angles are very sharp. The length of the leaf is 4.56 ± 0.99 cm (mean ± standard error), and its width is 1.09 ± 0.08 cm (mean ± SE). The tree has inflorescences with medium length. The fruits have an ovoid shape and are symmetric. Drupes sizes are ranging from medium to large (2-3 g), with an ovoid shape and an umbonate apex. The trees have limited vigor, not very voluminous foliage but with high density. Exhibits high oil yield with early oiling. Drupes have a high pulp-to-pit ratio and undergoes late and gradual veraison, with fruit color transitioning from light green to purplish-red. Pulp texture and strength remain high until fully ripened. The optimal harvest period is around mid-November. (www.assam.marche.it).

Figure 10: Olive cultivar Piantone Mogliano (Source: https://www.assam.marche.it)

1.6.4 Olive cultivar "Maurino"

Maurino (Figure 11), one of the most important cultivars in Central Italy, has low-to-medium vigor (Proietti *et al*., 2012). However, Parri *et al*. (2023) and Pasqualone *et al*. (2016) indicated that the Maurino cultivar is naturally occurring and has a historical presence in the Tuscany region. Trees are characterized by a limited vigor, featuring low-volume canopy, narrow elongated leaves, with a grayish-green color on the upper side. Both basal and apical angles are very sharp. The length of the leaf is 5.53 ± 0.64 cm (mean ± standard error), and its width is 0.90 ± 0.02 cm (mean ± SE). It has short inflorescences. The plants enters early in the physiological stage of fruit production. Maurino"s plant produces medium-sized (1.5-2 g), oval-shaped fruit, with a medium oil yield. The fruits exhibit a medium-to-high pulp-to-stone ratio. The fruit color ranges from green to light purple. The optimal harvest period is around the end of October to the beginning of November (www.assam.marche.it)

Figure 11: Olive cultivar Maurino (Source: https://www.assam.marche.it)

1.7 Propagation Methods

Plant propagation occurs through two main methods: sexual and asexual. In natural settings, plants predominantly propagate through sexual reproduction, producing viable seeds. Due to the combination of hereditary traits from two parents, plants coming from seedlings exhibit new and distinct characteristics from a genetic perspective. Seed propagation, across all cultivars, resets the new plant to its juvenile stage, characterized by the absence of fruit production for several years. The significance of obtaining propagation material through sexual methods could be useful for acquiring new rootstocks and utilizing them for genetic improvement (Fabbri *et al*., 2009; de Almeida Cançado *et al*. 2013; Sebastiani and Busconi, 2017). The asexual or "vegetative" method involves cell division in the mother plant, resulting in a daughter plant with identical genetic characteristics. Key techniques of asexual propagation include cuttings, layering, division, budding, and grafting. Cuttings entail rooting a severed section of the parent plant, layering involves rooting a portion of the parent before severing it, and budding and grafting entail joining two plant parts from different compatible genotypes (Fabbri *et al*., 2009; Bayraktar *et al*., 2020).

1.7.1. Traditional Propagation Techniques

In olive-growing countries, olive propagation is achieved by rooting of leafy stem or softwood cuttings, by grafting pieces of the stem (scions) onto seedlings or clonal rootstocks, or today only occasionally, by

regenerating whole plants from the ovules, that is, characteristic tissue hyperplasia that appears as protuberances at the collar of old trees (Fabbri *et al*., 2004, 2009). Among these techniques, rooting of leafy stem cuttings under mist is by far the most common technique that was first applied in America in the 1950"s (Fabbri *et al*., 2009).

In general, cuttings are obtained from one-year-old or younger shoots by dividing them into 10–15 cm pieces of 4–6 mm in diameter, with 4–6 nodes and with the 4–6 leaves at the distal nodes maintained on the cuttings. Several cultivars are hard to root or do not root at all. For this reason, the cutting should be treated at their basal ends with a root-promoting agent like auxins, mainly indole-3-butyric acid (IBA), afterward, the basal treated end of the cuttings should be inserted in rooting medium/substrate (perlite, peat moss) inside a rooting bench and maintained under mist conditions (Fabbri *et al*., 2009).

The method of grafting scions on seedlings is still used in Italy and in some "new" olive-growing countries, such as Argentina, where it has allowed a rapid diffusion of olive cultivation. In this technique, a short piece of shoot, mainly just one node, is grafted onto a rootstock that is developed from a seed (Fabbri *et al*., 2009).

Among the various traditional propagation techniques, the rooting of leafy stems has been widely employed since the mid-1950s. However, this method still presents several challenges. For instance: (a) successful rooting of cuttings is limited to spring (before blossoming) or the onset of autumn growth, (b) the capacity for adventitious rooting varies among cultivars, (c) even with similar parameters such as physiological, agronomical, and propagation conditions, different cultivars exhibit significant performance variations, and (d) in cultivars hard to root, grafting is the only viable technique for clonal propagation; however, propagation by grafting is more expensive, more complex and requires specialized nurseries and skilled grafters (Bayraktar *et al*., 2020). Grafting propagation, while used partially in certain countries like Italy due to high costs and a lack of selected rootstocks, is laborious and demands skilled grafters (Fabbri, 2006). Consequently, micropropagation emerges as an ideal tool for the clonal propagation of selected olive cultivars or clones (Lambardi and Rugini, 2003).

1.7.2. In Vitro Culture of Olive (Micropropagation)

Micropropagation is one advanced clonal propagation technique, regarding the application of tissue culture which allows the production of a considerable number of plants from small pieces of the mother plant in a relatively short period of time and limited space (Hassan and Zayed, 2018). Plant tissue culture or *in vitro* culture has been a remarkably successful technique in plant biotechnology. *In vitro* culture has been intensively studied and improved over the last six decades, which is now regularly adopted as a routine process in several plant species to accelerate the efficient clonal propagation of various commercial crops. Plants are propagated *in vitro* by cultivating stems, leaves, apical and axillary buds, immature seeds, embryos, and cotyledons. There were several factors controlling micropropagation of fruit trees such as explant type and size, surface sterilization, phenol exudation and its control, different culture media, carbon source and additives, light and temperature, plant growth regulators, pH (Hassan and Zayed, 2018). Due to its recalcitrant nature, tissue

oxidation, and challenges in obtaining sterile plant material and establishing shoot cultures, micro propagating economically important olive varieties is a difficult task (Lambardi *et al*., 2013; Yancheva and Kondakova, 2016). The success of olive micropropagation is highly dependent on the cultivar, with generally low shoot proliferation rates, difficulty in rooting micro propagated olive cultivars, and a high rate of post-transplanting losses (Grigoriadou *et al*., 2007; Sanchez-Romero, 2018). However, unlike many fruit species, at the beginning of the 1990s only a few olive cultivars have been efficiently propagated *in vitro* by micropropagation (Rugini and Fedeli, 1990; Fabbri *et al*., 2009). Moreover, at that time, micropropagation was often initiated using explants from embryos and seedlings (Bao *et al*., 1980; Fabbri *et al*., 2009), but this approach is of minor interest when reproducing selected cultivars or clones (Fabbri *et al*., 2009).

Advantages of the adaptation of micropropagation in the clonal olive propagation are (Pollastri, 2008; Hassan and Zayed, 2018; Regni *et al*., 2022):

- A vast number of plants can be cultivated from a single individual through clonal propagation.
- This method facilitates the growth, storage, and maintenance of a large quantity of plants in limited spaces, making it a cost-effective process.
- It can be carried out throughout the entire year, irrespective of the season.
- This technique applies to many genotypes where *in vivo* plant multiplication is challenging or even impossible.
- Micro-propagated plants are in general more uniform and can be produced in less time compared to traditional methods.
- Possibility to ascertain the genetical and sanitary health status of the micro-propagated plant also through remediation and sanitation from pathogens.
- Plants developed from virus-free stock are immune to re-infection, and the micro-propagated plants can be transferred without quarantine, ensuring the new plants produced are disease-free.
- Due to the more precise control of nutrition, growth regulator levels, temperature, and light in cultures, the rate of multiplication in vitro is generally much faster than in vivo techniques of vegetative propagation.

The disadvantages related to micropropagation are:

- Establishing and equipping a laboratory for tissue culture can demand additional labor and incur higher costs due to the need for specialized instruments and chemicals.
- Tissue culture is an advanced technique that necessitates a certain level of knowledge and proficiency for individuals entering the field.
- Contamination poses a significant challenge in tissue culture settings, where plants are susceptible to infections by bacteria, fungi, and viruses (Figure 12). Therefore, stringent measures and the use of personal protective equipment (PPE) are essential precautions when conducting tissue culture in the laboratory.

- In addition, the high initial costs, and the probable onset of somaclonal variations in the micro-propagated material are other factors to take into consideration.

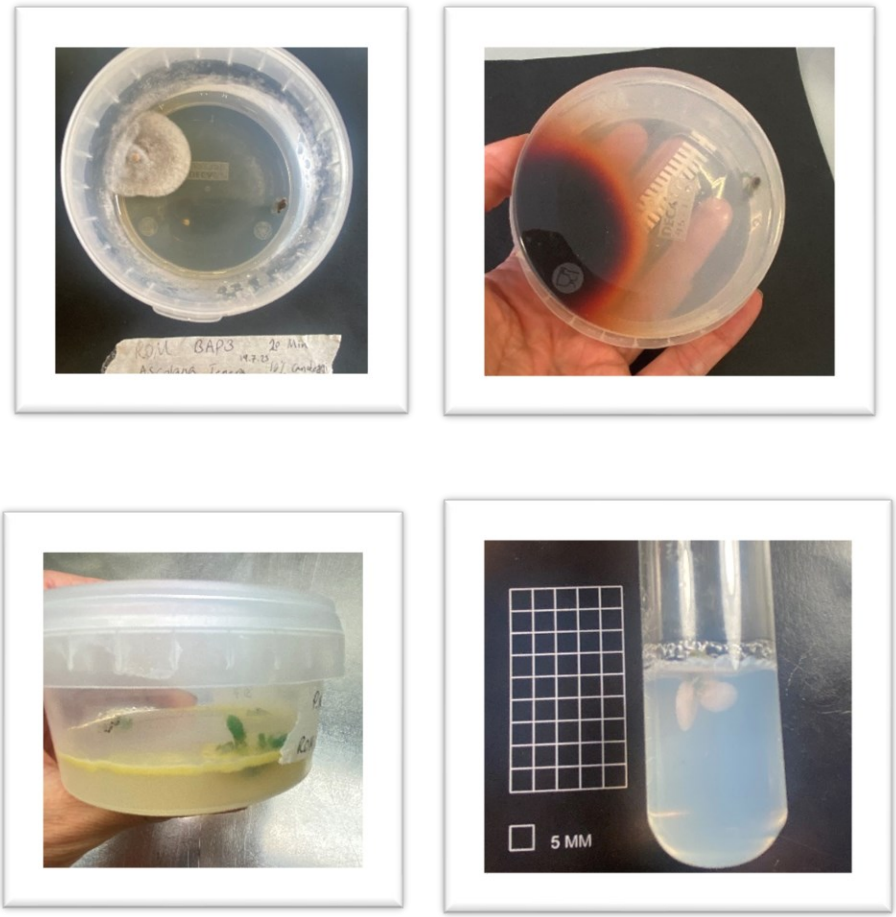

Figure 12: Contaminated sample.

- The material must undergo screening before being cultured; overlooking any abnormalities in the process could result in the new plants becoming infected.
- This technique is not universally applicable to all crops, and certain cultivars may pose challenges for *in vitro* growth.

1.8 The aim of study

The study aims to advance *in-vitro* propagation techniques for sterilization and proliferation of local olive varieties, including 'Ascolana Tenera,' 'Piantone di Mogliano,' 'Piantone di Falerone,' and 'Maurino.' The key objectives include the optimization of sterilization protocols, through the test of several concentrations and timing of exposure of two sterilizing agents, avoiding the use of the toxic compound mercurium chloride, one of the most used to sterilize olive explants. The best combination of the concentration and exposition time serves to eliminate contaminants while preserving the vitality of the explant. One of the main points of the propagation of the olive tree is the maintenance of genetic integrity, pioneering innovative *in vitro* proliferation protocols tailored to each cultivar, and exploring cultivar-specific responses, aiming to produce plants considered clone of the mother plant. Eight different proliferation media have been tested in order to select the best PGRs type and concentration, able to enhance shoot proliferation, in the olive genotypes that are mainly considered recalcitrant to *in vitro* regeneration. The proliferation studies performed and described in this thesis on the culltivar Piantone di Mogliano are a good starting point for future improvement of proliferation rate and to develop a whole *in vitro* process that terminate with an acclimatized plant in the greenhouse.

The broader significance lies in contributing to the conservation and propagation of local olive varieties from the Marche region and central Italy.

CHAPTER 2
MATERIAL AND METHODS

The experiment was conducted at the Plant Biotechnology Laboratory of the Department of Agricultural, Food, and Environmental Science at the Marche Polytechnic University. In this study, we developed an *in-vitro* micropropagation protocols for two stages of sterilization and proliferation in 3 local olive cultivars from the Marche region, namely, "Ascolana Tenera", "Piantone Mogliano", and "Piantone Falerone". Additionally, the same sterilization procedures has been attempted in "Maurino" cultivar from central Italy.

2.1 Instruments

Table 5 shows the list of instruments used in the laboratory. Additionally, Figure 13 displays several instruments. The containers used for cultivating plant material included tubes, micro boxes, culture vessels, and Petri dishes. An autoclave was employed for sterilizing containers, tubes, culture medium, and water. Laminar flow hoods were utilized to protect the working environment from dust and airborne contaminants by maintaining a constant, unidirectional flow of HEPA-filtered air over the work area. For the initiation of the proliferation stage, a growth chamber was used with a photoperiod of 16 hours, maintaining optimum temperature (25±3°C), relative humidity (60 to 80%), and controlled light (1500-2000 lux), respectively. A magnetic mixer combined with a hot plate was used with 0.5 L, 1 L, or 2 L glass bottles, glass beakers, burettes, measuring cylinders, volumetric flasks, and a tube rack. Pipettes were employed for transporting measured volumes of liquid. Various scalpels with replaceable blades, forceps, a mini high-temperature sterilizer box, and tools for disinfection were also utilized. Additionally, a laboratory water distiller was part of the equipment, which produced distilled ultra-pure water, necessary for media preparation.

Table5. List of instruments used in the laboratory.

Description	Manufacturer
Containers (tube, micro box, culture vessel, Petri dish)	AuChem instruments
Autoclave	Fedegari
pH-meter	AuChem instruments
laminar flow hood	Cavallo
Growth chamber	Various manufacturers
Magnetic mixer	AuChem Instruments
Pipet	StarLab
Laboratory water distiller	Sigma
Analytical digital balance	Alessandrini

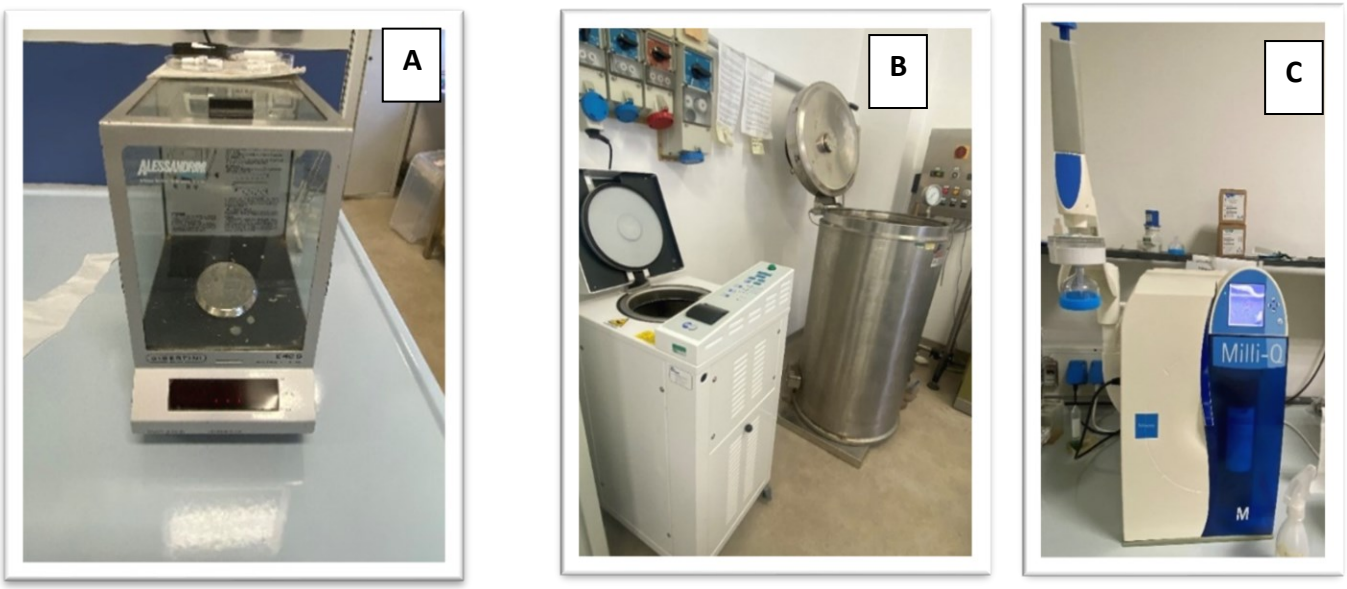

Figure 13: Digital Balance (A); Autoclave (B); Water distiller (C).

2.2. Plant Materials

Olive explants were obtained from the experimental farm of the Marche Polytechnic University (Figure 14), located in Agugliano (Marche, Italy), from four self-rooted olives (*Olea europaea* L.) cultivars of "Ascolana Tenera", "Piantone di Mogliano", "Maurino" and "Piantone di Falerone". These trees were planted

with a spacing of 4 × 2 m (1250 tree ha^{-1}) in May 2012 and were approximately 12 years old at the time of the experiment. The experimental farm contains native, national, and international cultivars.

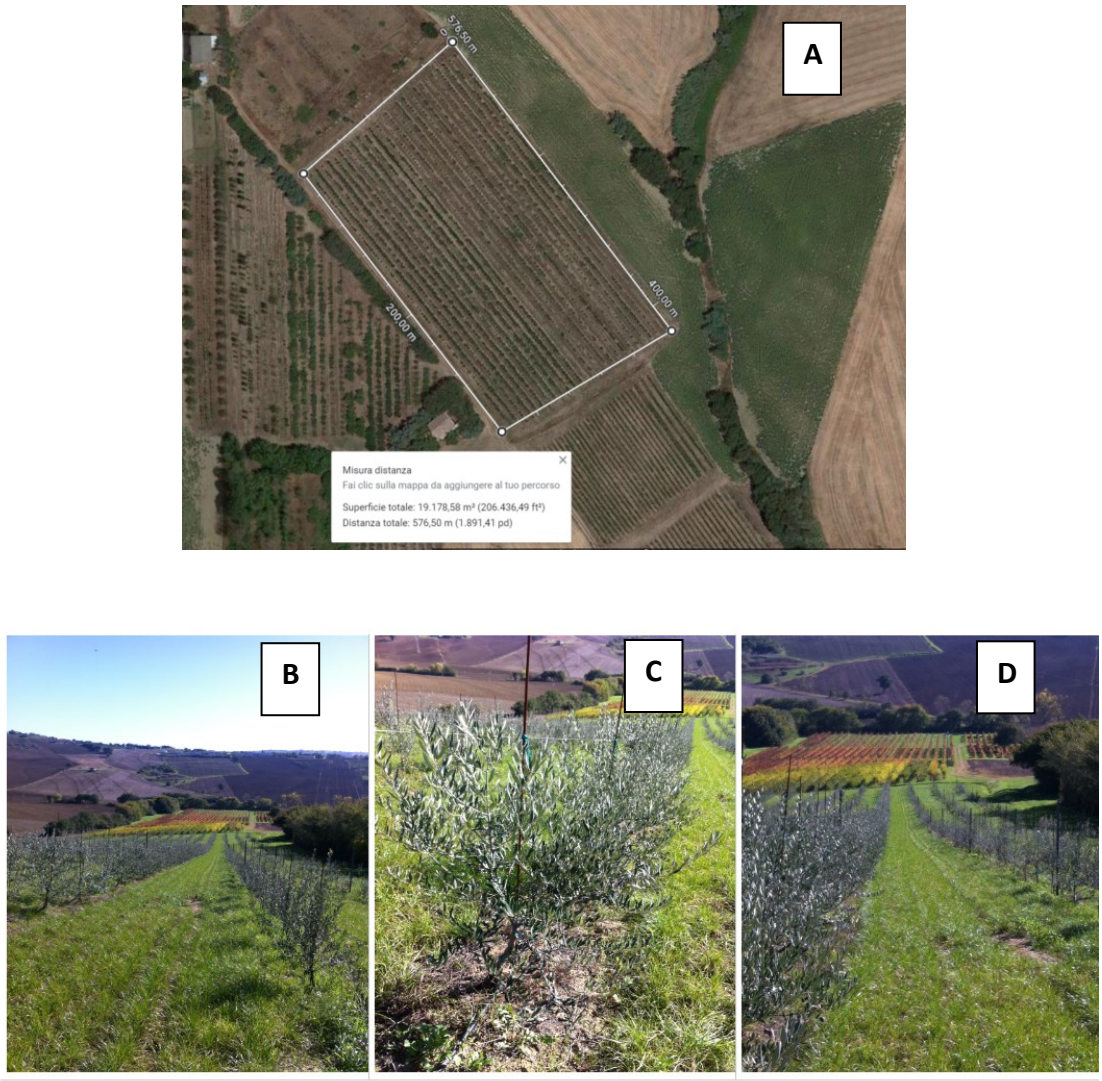

Figure 14: The experimental farm of the Marche Polytechnic University; Aerial photo (A) (Source: Google Maps); Several rows of olive groves (B to D).

Experiments utilized newly sprouted, non-lignified shoots that have been withdraw from these olive plants as the source of starting material for the sterilization phase (Figure 15). For the culture initiation, single nodal explants, each approximately 1.5-2.0 cm in size and containing two opposite buds, were meticulously prepared removing all the existing leaves. All buds of the shoots, including the apical bud segment were sampled (Figure 16). Similar method also was employed by Bayraktara *et al* (2020), however their shoot was segmented between the third and sixth nodes of vigorously growing shoots.

Figure 15: Example of the shoot that has been used as a plant material related to olive cultivar “Maurino”

Figure 16: Example of explant that has been prepared for olive cultivars; “Piantone Mogliano” (A); “Maurino” (B); “Ascolana Tenera” (C); “Piantone Falerone” (D).

The plant material has been collected from the olive orchard starting at the beginning of March 2023 until mid-July 2023, with an increase in the frequency of plant material harvesting in May and June.

2.3. Sterilization

Sterilizing tissue before introducing explants *in vitro* is a crucial step in olive micropropagation (Lambardi and Rugini, 2003). Additionally, during this initial phase, it is imperative not only to chemically disinfect the plant material but also to observe hygienic practices for both individuals and equipment (cleaning of hands and work surfaces with disinfectant or alcohol ethyl, cleaning of instruments with mini high-temperature sterilizer).

In the first step the uni-nodal shoot segments explants, were pre-washed under running tap water. In the next step, explants have been washed with fungicide. The explants were then washed for 30 minutes in a

solution containing distilled water and Previter (10.0 mg/L), (ARYSTA) a systemic fungicide based on Propamocarb (Chemical family: Carbamates; Mechanism of action: FRAC 28), using a magnetic mixer.

The sterilization procedures were followed by using 70% ethanol (C_2H_6O, EtOH 99.9%) solution for 1 minute.

In the last step of sterilization, the plant surface was disinfected using sodium hypochlorite (Clorox, NaClO) (MONDIAL S.R.L), with the solution containing 3 drops of Tween 20 (Sigma-Aldrich). Throughout 12 experiments of sterilization, NaClO have been used with varying percentages and time of exposition with the sterilization agent to achieve the maximum survival percentage of axillary buds and explant segments (Table 6). Furthermore, the nodal explants were thoroughly washed three times with sterile distilled water (each time for 5 minutes).

Table 6. Varying percentages of NaClO and different durations were employed to achieve the maximum survival percentage.

Treatment	Sodium Hypochlorite (NaClO)	Time (Min)
1	25	30
2	25	15
3	20	15
4	20	10
5	15	15
6	15	10
7	15	05
8	10	30
9	10	20
10	10	15
11	10	10

In each experiment, the pH of the culture medium was adjusted to 5.7-5.8 with 1M KOH before the autoclave sterilization cycle at 121°C for 20 minutes, using professional pH meter (Hana Instrument). Subsequently, the culture media were poured into sterile glass tubes (each containing approximately 10 mL). Both ends of the explants were cut off using a scalpel and transferred to a culture tube. This process was conducted under a laminar flow hood in an aseptic environment.

Nodal explants were allowed to grow under direct light in a growth chamber with a 16-hour photoperiod at a light intensity of 70 µmol/m2/s and a temperature of 24°± 1°C, maintaining a relative humidity of 60 to 80%. Over 7 days, observations were conducted daily to record data, including monitoring for symptoms of infection or contamination by fungi and bacteria. Data acquisition on the number of contaminated, necrotic, and vital explant has been acquired for each genotype and each sterilization treatment applied, seven days after the sterilization trial. All these data have been reported as percentage on the total of explants treated. Furthermore, the source of contamination (bacterial or fungal) has been noted for each sterilizing treatment. Subsequently, the explants were transferred to a fresh culture medium.

2.4. Initial *in vitro* proliferation phase

In the initial phase, the sterilized explants were cultured on Rugini Olive Medium (ROM) (Duchefa, Biochemie). Table 7 shows the chemical component of ROM.

Table 7. The chemical composition of Ruggini Olive Medium. (modified from: www.duchefa-biochemie.com)

Micro Elements	**mg/l**	**µM**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.25	1.00
FeNaEDTA	36.70	100.00
H_3BO_3	12.40	200.55
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	14.30	49.75
Macro Elements	**mg/l**	**mM**
$CaCl_2$	332.16	2.99
$Ca(NO_3)2$	416.92	2.54
KCl	500.00	6.71
KH_2PO_4	340.00	2.50
KNO_3	1100.00	10.88
$MgSO_4$	732.60	6.09
NH_4NO_3	412.00	5.15
Vitamins	**mg/l**	**µM**
Biotin	0.05	0.20
Folic acid	0.50	1.13
Glycine	2.00	26.64
myo-Inositol	100.00	554.94
Nicotinic acid	5.00	40.62
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.50	1.48

Total concentration Micro and Macro elements including vitamins: 4023.89 mg/l

In addition to Rugini olive media basal salt and vitamins, media preparation regarded the supplementation of 30 g/L of sucrose, 7 g/L of plant agar (Duchefa Biochemie), Plant Growth Regulators (PGRs) added before autoclave (in case of 6-Benzyl-aminopurine-BAP) or after the autoclave sterilization prior to filter sterilization in the aseptic environment (nylon mesh filters, with pore at 0.45 μm).

One week after the transfer on ROM medium, the explants have been checked for the detection of contamination. Contaminated samples will be discarded following specific protocols, while the remaining samples have been prepared for the proliferation phase.

2.5. Proliferation

During the multiplication phase, the previously initiated explants have been sub-cultured onto a new fresh culture medium containing specific growth regulators, such as cytokinin, which stimulate cell division and shoot formation. This leads to the production of multiple shoots from a single explant. These new shoots can then be further separated to generate an increased number of identical plantlets (Wong *et al*., 2021; Darwesh *et al*., 2022). The proliferation medium has been tailored for each genotype, identifying the concentration of plant growth regulators that induces the highest proliferation rate. To determine a suitable medium for the proliferation of olives, two distinct experiments were conducted. In the first set, explants were cultured in five proliferation media (P1, P2, P3, P4, P5) with different combinations of cytokinin: 6-benzylaminopyrine (BAP) and Zeatin, as outlined in Table 8.

Table 8. Concentration of plant growth regulators for the first set of experiments.

Medium name	**BAP (mg/L)**	**Zeatin (mg/L)**
P1	0	3
P2	1	2
P3	2	1
P4	3	0.5
P5	0	0

Figure 17: Micro boxes contain the first set of substrates as outlined in Table 5.

In the second set of experiments, only the plant growth regulator BAP was applied, starting from the addition of 3 mg/L of BAP (P6), and testing increasing BAP concentration on P7 and P8, having 4 and 5 mg/L, respectively and following the specification reported in Table 9. Proliferation results for both sets of media were assessed for a total of nine weeks, transferring, and acquiring data every three weeks of culture.

Table 9. Concentration of plant growth regulators for the second set of experiments.

Medium name	**BAP (mg/L)**	**Zeatin (mg/L)**
P6	3	0
P7	4	0
P8	5	0

For each proliferation media, at each sub-culture (three in total), the percentage of contaminated and necrotic explant have been reported. The average number of shoots, the average number of shoot primordia, the average number of shoots nodes and the average number of lateral shoots have been used as important parameter to determine the efficiency of shoot production for each proliferation medium tested.

2.6. Statistical analyses

All acquired data from each sterilization and proliferation trial were analyzed by one-way ANOVA, and the Duncan test ($p < 0.05$) was used to identify significant differences among the different parameters analyzed. The bars depicted in the graphs represent the standard error derived from the standard deviation of the values.

CHAPTER 3
RESULTS AND DISCUSSION

The experimental trial, regarding the optimization of the *in vitro* sterilization and proliferation protocols conducted on the four olive cultivars: “Ascolana Tenera”, “Piantone di Mogliano”, “Piantone di Falerone”, and “Maurino” have been reported with the aim to find the best combination of time of exposure and concentration of the sterilizing agent, which allowed the obtainment of maximum number of healthy plants able to adapt to *in vitro* culture conditions. Furthermore, the use of different PGRs allowed to define the best combination and concentration of cytokinins, able to enhance *in vitro* shoot proliferation.

3.1. Sterilization

To determine an efficient sterilization solution and method, nodal explants of four different olive cultivars (Ascolana Tenera, P. Falerone, P. Mogliano, and Maurino) were sterilized in 11 different solutions of sodium hypochlorite, each with varying concentrations and exposure times. Additionally, one treatment involved the use of hydrogen peroxide for a duration of 3 hours (see material and method). After sterilization, nodal explants were cultured in a ROM culture medium containing 3 mg/L of BAP for three weeks in a growth chamber at 25°C. Data collection occurred on the seventh day after the various sterilization treatments.

3.1.1 Ascolana Tenera

In the cultivar Ascolana Tenera, the number of explants, along with the associated sterilization methods specifics (including time and percentage of sodium hypochlorite), as well as details concerning healthy, contaminated, and necrotic explants has been reported for each treatment and presented in Table 10.

Table 10: Sterilization results for the olive cultivar “Ascolana Tenera”.

Sterilization treatment	Sodium Hypochlorite (Percentage)	Time of sterilization (min)	Explant (no)	Healthy Explant (no)	Explant Contaminated by fungi (no)	Explant Contaminated by bacteria (no)	Necrotic Explant (no)
1	25	30	40	0	35	2	3
2	25	15	40	0	36	0	4
9	10	20	95	0	12	83	0

In this cultivar, three sterilization treatments have been applied, the first two using a concentration of sodium hypochlorite of 25% for 15 and 30 minutes, and the last with 10% of sodium hypochlorite for 20 minutes. Seven days after the sterilization treatment, no healthy explants were observed after examining three different sterilization methods. There was no significant difference between treatments 1 and 2 in terms of contamination and necrosis. However, treatment 9 exhibited notable distinctions from both (Figure 18).

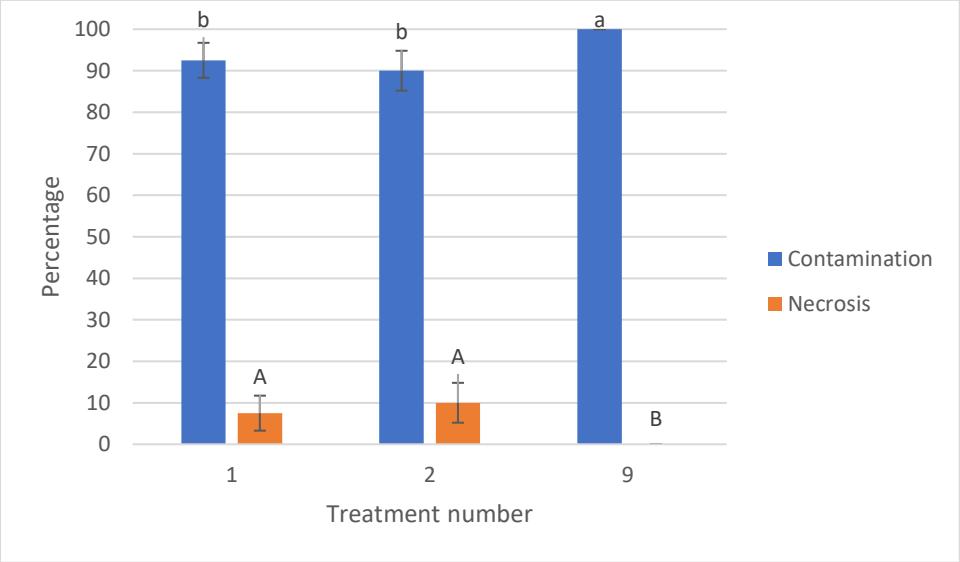

Figure 18: Comparison of contamination and necrosis levels in olive cultivar “Ascolana Tenera” across three sterilizing-treatment conditions. Small letters represent differences in the percentage of contaminated explants between the different treatment, capital letters represent the differences in the percentage of necrotic explants. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 32$).

With respect to the contamination source, fungi were identified as the main source in treatments 1 and 2, while bacteria emerged as the primary contaminant in treatment 9 (Figure 19).

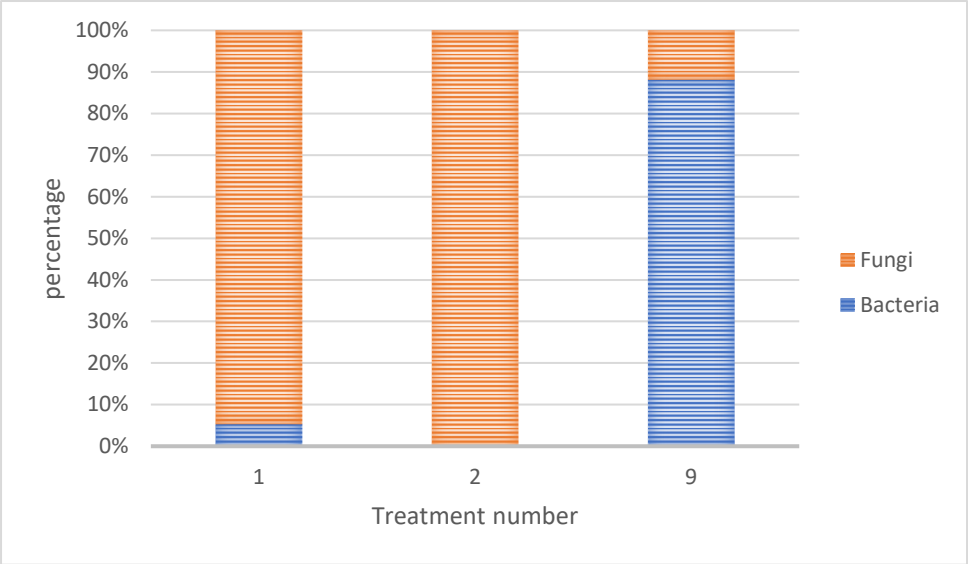

Figure 19: Comparison of contamination sources in olive cultivar “Ascolana Tenera” across three treatment conditions.

3.1.2 Piantone di Falerone

In the Piantone di Falerone cultivar, the report provides information on the number of explants, details about the associated sterilization methods (including duration and percentage of sodium hypochlorite), and specifics regarding the health status of explants (healthy, contaminated, and necrotic) for each treatment. This data is presented comprehensively in Table 11.

Table 11: Sterilization results for the olive cultivar “Piantone di Falerone”.

Sterilization treatment	Sodium Hypochlorite (Percentage)	Time of sterilization (min)	Explant (no)	Healthy Explant (no)	Explant Contaminated by fungi (no)	Explant Contaminated by bacteria (no)	Necrotic Explant (no)
1	25	30	40	0	36	2	2
2	25	15	40	0	36	0	4
3	20	15	117	0	86	0	31
5	15	15	72	29	33	0	1
6	15	10	32	7	16	0	10
7	15	5	32	2	19	0	11
11	10	10	32	2	22	0	8
12	Hydrogen peroxide H_2O_2 3%	180	56	0	27	1	28

Among the 8 sterilization treatments, healthy explants were observed in 4 treatments. The highest proportion of healthy explants was observed in treatment 5, Characterized by a 15 % of sodium hypochlorite and a sterilization time of 15 minutes. Furthermore, the data indicated that there was no survival rate of explants in sterilization treatments with a percentage of sodium hypochlorite higher than 20 percent. A significant difference in the number of healthy explants have been observed between treatments 5 and 6 compared to the other treatments (as reported in Figure 20). Additionally, the number of healthy explants differs between treatment 5 and 6.

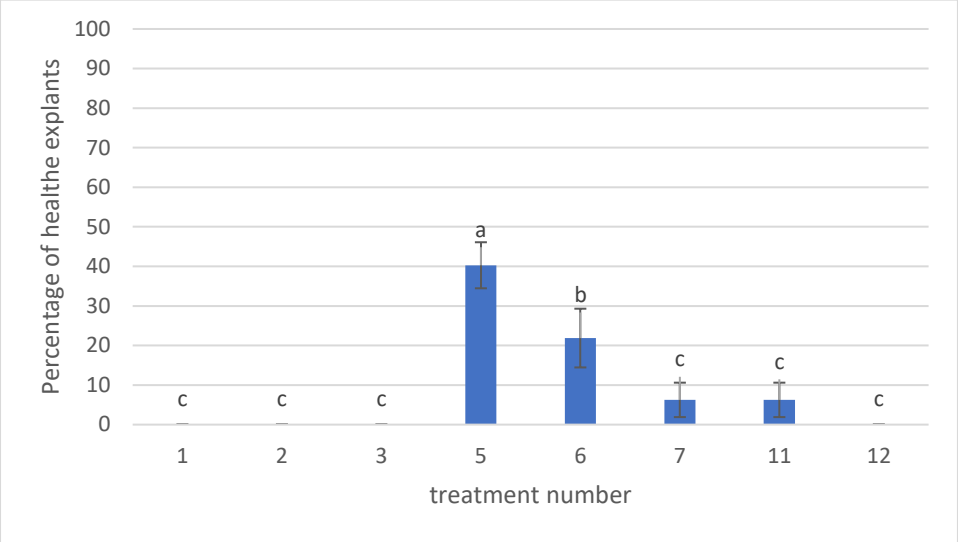

Figure 20: Comparison of healthy explants in olive cultivar “Piantone di Falerone” across eight sterilizing-treatment conditions. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 32$).

The highest percentage of contamination has been observed in treatments 1 and 2, while the lowest percentages have been observed in treatments 5, 6, 7, and 12. In terms of necrotic explants, the highest percentages have been attributed to treatments 12 and 7, whereas the lowest percentages have been observed in treatments 1, 2, and 5. Figure 21 illustrates differences between treatments in terms of contamination and necrosis.

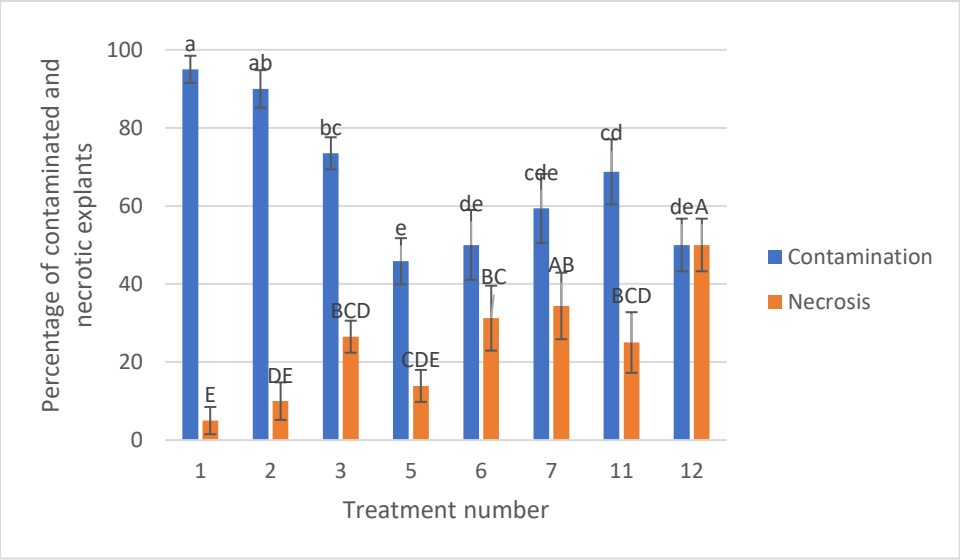

Figure 21: Comparison of contamination and necrosis levels in olive Cultivar "Piantone di Falerone" across eight sterilization-treatment conditions. Small letters represent differences in the percentage of contaminated explants between the different treatment, capital letters represent the differences in the percentage of necrotic explants. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 32$).

In all eight sterilization treatments, the mainly contamination source was identified as fungi; however, treatments 1 and 12 exhibited a small percentage of contamination attributed to bacteria (Figure 22).

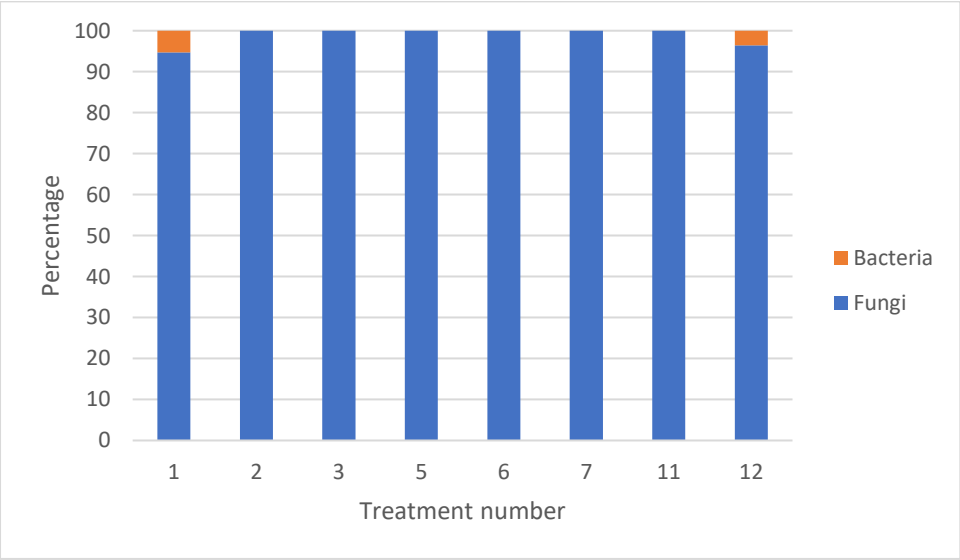

Figure 22: Comparison of contamination sources in olive cultivar "Piantone di Falerone" across eight sterilizing-treatment conditions.

3.1.3 Piantone di Mogliano

In the Piantone di Mogliano cultivar, the number of explants, along with detailed information on sterilization methods (including the duration and percentage of sodium hypochlorite), as well as specifics regarding healthy, contaminated, and necrotic explants, have been recorded for each treatment. This data is presented in Table 12.

Table 12. Sterilization results for the olive cultivar "Piantone di Mogliano".

Sterilization treatment	Sodium Hypochlorite (Percentage)	Time of sterilization (min)	Explant (no)	Healthy Explant (no)	Explant Contaminated by fungi (no)	Explant Contaminated by bacteria (no)	Necrotic Explant (no)
1	25	30	40	0	37	0	3
2	25	15	120	0	50	1	69
3	20	15	47	0	47	0	0
5	15	15	124	75	27	0	22
6	15	10	72	26	20	0	26
8	10	30	40	14	17	0	9
10	10	15	69	22	22	1	24
12	Hydrogen peroxide H_2O_2 3%	180	48	0	19	0	29

Among the 8 sterilization treatments, healthy explants were observed in 4 treatments. The highest proportion of healthy explants was observed in treatment 5, characterized by 15 % sodium hypochlorite and a sterilization time of 15 minutes, a similar condition observed in the cultivar Piantone di Falerone. Furthermore, the data indicated that there was no survival rate of explants in sterilization treatments with a percentage of sodium hypochlorite higher than 15 percent.

A significant difference in the number of healthy explants between treatment 5 compared to the other treatments has been reported for this cultivar, as illustrated in Figure 23. Although lower level of survival rate has been reached, also treatments 6, 8, and 10 led to the successful obtainment of at least 30% of healthy explants.

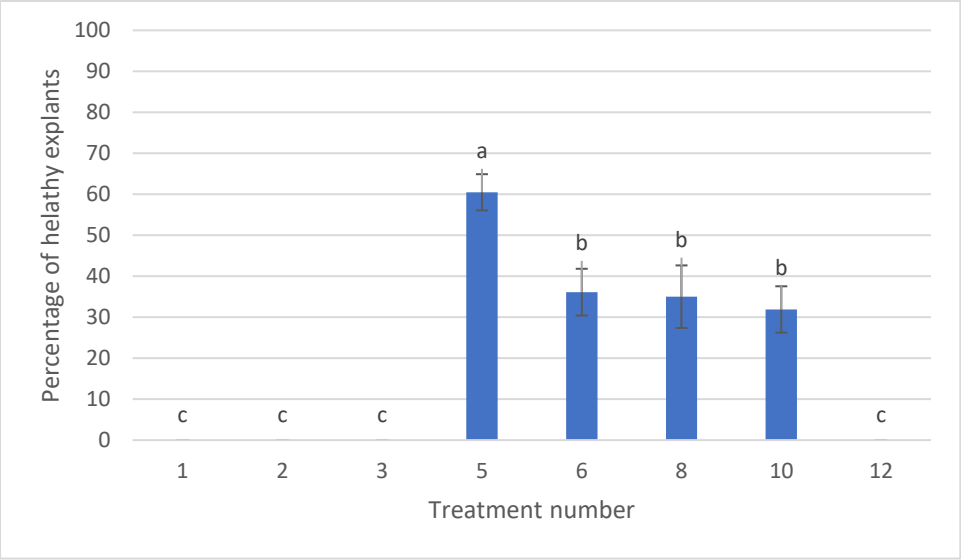

Figure 23: Comparison of healthy explants in olive cultivar "Piantone di Mogliano" across eight sterilizing-treatment conditions. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 32$).

In regard to the contamination level, the highest percentage of contamination have been associated with treatments 1 and 3, while the lowest percentages have been observed in treatments 5, 6, and 10. In terms of necrotic explants, the highest percentages have been attributed to treatments 12 and 2, whereas the lowest percentages have been observed in treatments 1 and 3. All contamination and necrosis results has been acquired 7 days after the sterilization trial, and the differences have been reported in Figure 24.

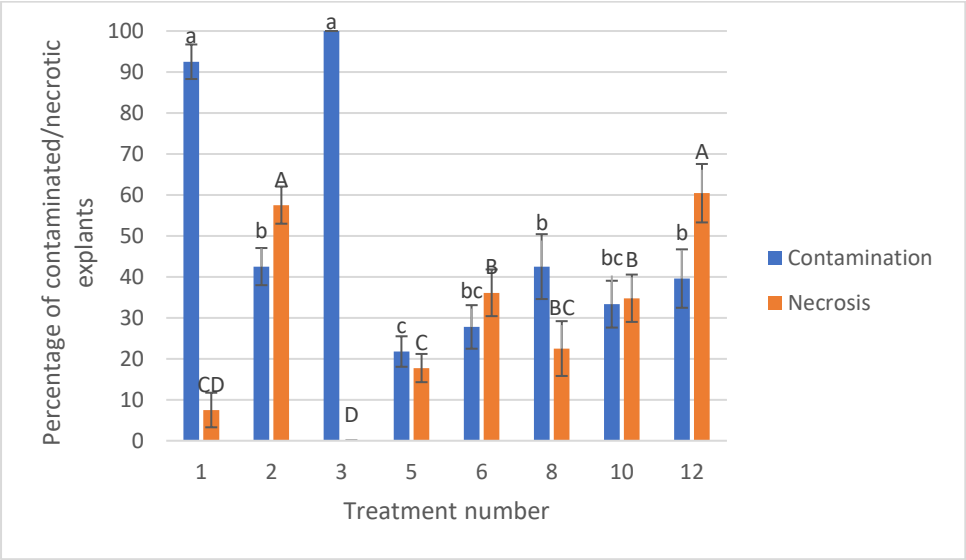

Figure 24: Comparison of contamination and necrosis levels in olive cultivar "Piantone di Mogliano" across eight sterilizing-treatment conditions. Small letters represent differences in the percentage of contaminated explants between the different treatment, capital letters represent the differences in the percentage of necrotic

explants. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 32$).

In all eight sterilization treatments, the main contamination source was identified as fungi; however, treatments 2 and 11 exhibited a small percentage of contamination attributed to bacteria (Figure 25).

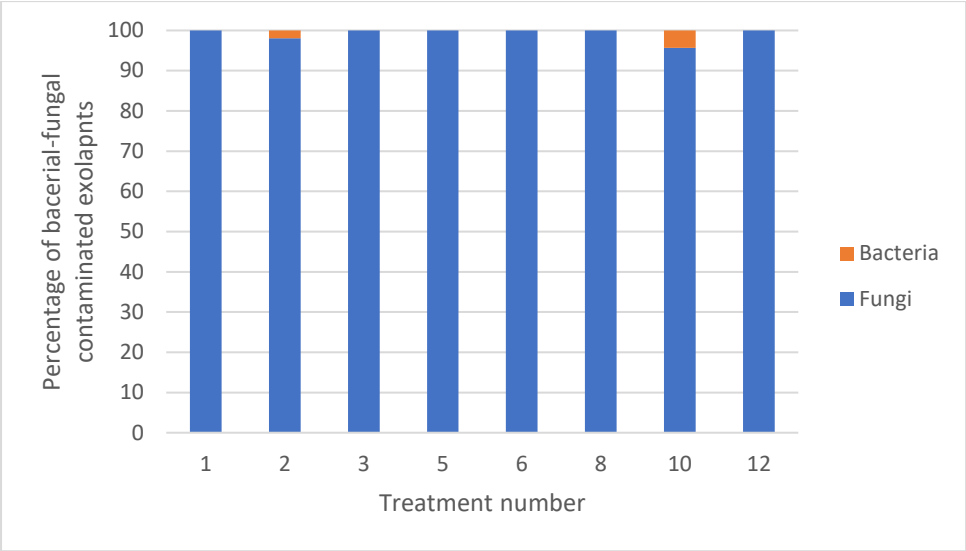

Figure 25: Comparison of contamination sources in olive cultivar “Piantone di Mogliano” across eight sterilizing-treatment conditions.

3.1.4 Maurino

For the Maurino cultivar, the quantity of explants, along with the specific details of sterilization methods (including duration and concentration of sodium hypochlorite), as well as information regarding healthy, contaminated, and necrotic explants, have been documented for each treatment. These details are outlined in Table 13.

Table 13. Sterilization results for the olive cultivar “Maurino”.

Sterilization treatment	Sodium Hypochlorite (Percentage)	Time of sterilization (min)	Explant (no)	Healthy Explant (no)	Explant Contaminated by fungi (no)	Explant Contaminated by bacteria (no)	Necrotic Explant (no)
1	25	30	40	0	22	2	16
2	25	15	120	0	79	3	38
3	20	15	91	3	61	0	27
4	20	10	32	5	11	0	16
5	15	15	117	24	68	0	25
6	15	10	168	40	103	8	17
10	10	15	39	13	12	10	4
11	10	10	42	10	30	0	2
12	Hydrogen peroxide H_2O_2 3%	180	48	0	17	0	31

Among the 9 sterilization treatments, healthy explants were observed in 6 treatments. The highest proportion of healthy explants was observed in treatment 6, characterized by 15 % of sodium hypochlorite and a sterilization time of 10 minutes. Furthermore, the data indicated that there was no survival rate of explants in sterilization treatments with a percentage of sodium hypochlorite higher than 25 percent.

No significant difference in the number of healthy explants have been detected among treatments 5, 6, 10, and 11. Notably, the highest percentage of healthy explants, have been cultured on treatment 10 (33.33%), followed by treatments 11 (25%), 6 (23.8%), and 5 (20.51%) not statistically different to the treatment 10 (Figure 26). With a percentage of healthy explants of 15.63%, treatment 4 stands out from the remaining treatments which failed to preserve the vitality of the explants.

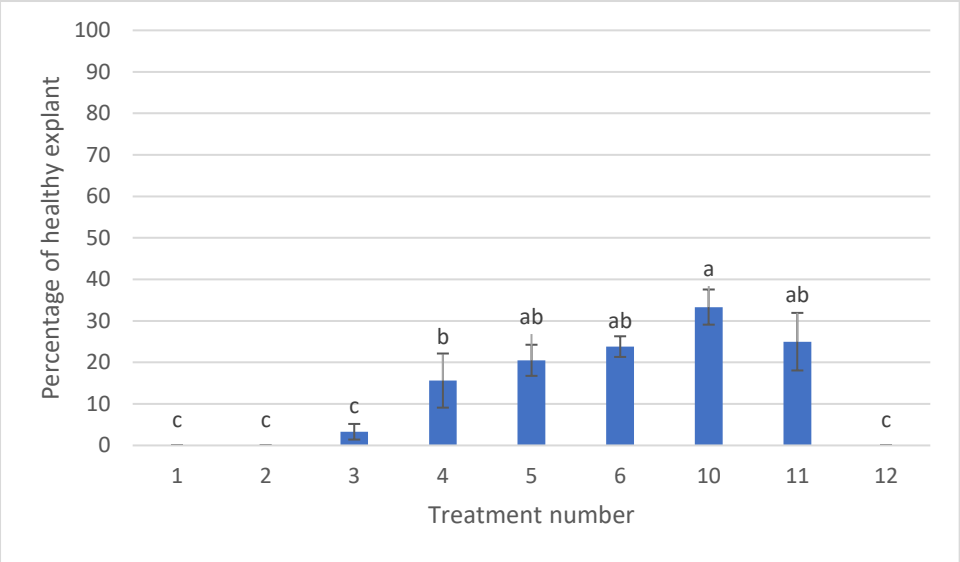

Figure 26: Comparison of healthy explants in olive cultivar "Maurino" across nine sterilizing-treatment conditions. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 32$).

In regards to bacterial and fungal contamination, the highest percentages of contamination have been associated with treatments 1, 2, 3, 5, 6, 10 and 11, while the lowest percentages have been observed in treatments 4, and 12. In terms of necrotic explants, the highest percentages have been attributed to treatments 12 and 4, whereas the lowest percentages have been observed in treatments 5, 6, 10 and 11 (Figure 27).

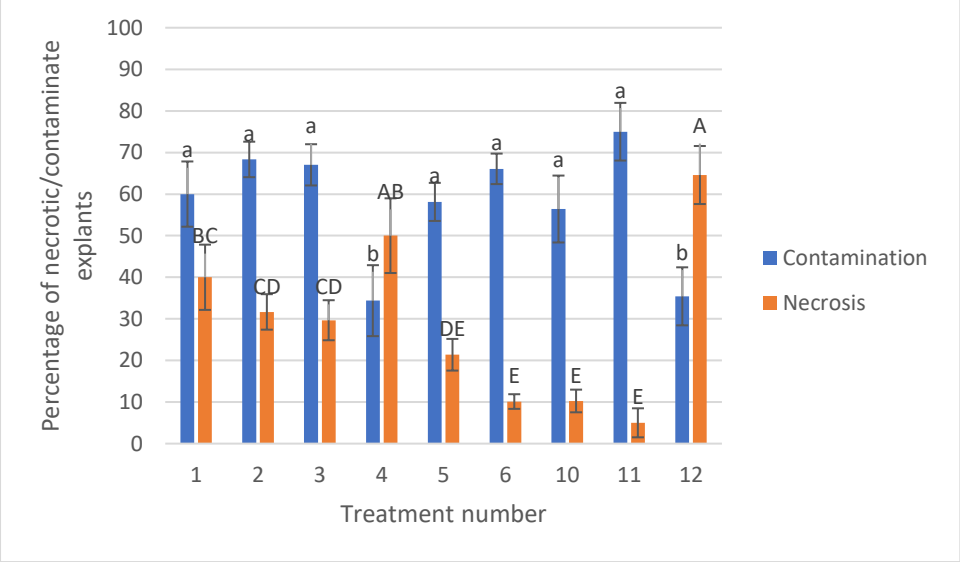

Figure 27: Comparison of contamination and necrosis levels in olive cultivar "Maurino" across nine-sterilizing-treatment conditions. Small letters represent differences in the percentage of contaminated explants between the different treatment, capital letters represent the differences in the percentage of necrotic explants. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 32$).

In eight out of the nine sterilization treatments, the main contamination source was identified as fungi; however, treatments 1, 2, and 6 exhibited a small percentage of contamination attributed to bacteria (Figure 28). The only exception was observed in treatment 10, where the percentage of contamination caused by bacteria was around 45%.

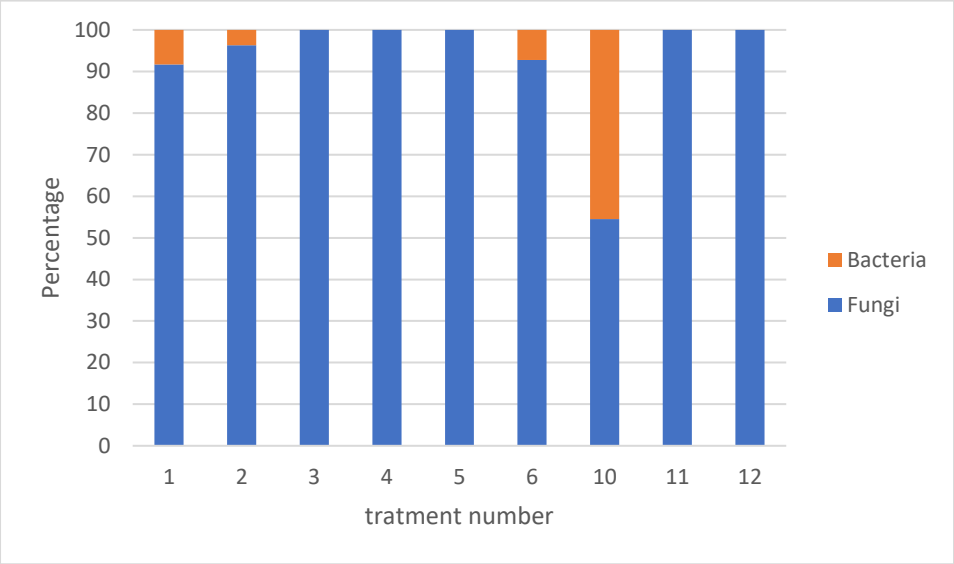

Figure 28: Comparison of contamination sources in olive cultivar "Maurino" across nine sterilizing-treatment conditions.

3.1.5 Concluding Sterilization Outcomes

Across all cultivars, fungal contamination was the dominant issue. Regarding the relationship between healthy explants and the percentage of sodium hypochlorite concentration, the cultivar "Maurino" exhibits the highest resistance to high concentrations of sodium hypochlorite, followed by the cultivars "Piantone di Falerone and "Piantone di Mogliano". In the case of "Ascolana Tenera", due to the absence of healthy explants, it was not possible to analyze this parameter. The application of hydrogen peroxide (H_2O_2) with a concentration of 3% for sterilization was not effective, as the majority of the explants necrotized after the treatment, maybe for the prolonged exposition time to the sterilization agent; in fact, after its application on three cultivars, "Maurino", "'Piantone di Falerone", and "Piantone di Mogliano", no healthy explants were observed. Based on our results, the highest efficiency in the sterilization protocol was achieved with treatment number 5 (15% of sodium hypochlorite for 15 minutes), followed by treatment 6 (15% of sodium hypochlorite for 10 minutes).

The adaptation to olive explants grown in the field to the *in vitro* conditions and the establishment of the sterile culture is one of the most challenging steps for the *in vitro* propagation of this recalcitrant species. Many publications in the scientific literature, report the use of mercuric chloride ($HgCl_2$) mainly used for less than 2 minutes at a 0.1% (v/w) as surface sterilization agent (Rostami et al., 2009; Grigoriadou et al., 2002). But this

mercuric salt, also given its high solubility and corrosivity, is considered highly toxic, with a lethal dose that ranged between 1 and 4 g if ingested (Beastely et al., 2014) and for this reason the use of this compound was not recommendable in an *in vitro* culture experimental laboratory. Sodium hypochlorite have been used as the main surface sterilizing agents, after a fungicide pre-treatment on Italian cultivars Coratina, Leccino, Nocellara and Pendolino, and the 20% of Bleach for 5 minutes was the treatment that maximize the survival rate and minimize tissue browning (Amhad et al., 2016). In the Greek cultivar Koroneiki, researchers defined as suitable a sterilizing solution of 10% of sodium hypochlorite for 10 minutes, with few drops of Tween 20, practically correspondent to the sterilization treatment 10 used in this study (Roussos et al., 2002). Piantone di Falerone and Piantone di Mogliano have been successfully established in the sterile *in vitro* condition through a sterilizing treatment including a mixture of sodium hypochlorite and hydrochloric acid (30:1 v/v) recording survival rates averaging slightly above the 20%, contamination rates equal to 30% and percentages of death explants just below the 50% (Varlaro et al., 2007). Our best performant sterilization procedures 5 for both the cultivars mentioned above, led to the obtainment of a 40% and 60% survival rate for Piantone di Falerone and Piantone di Mogliano respectively, with contamination and necrosis rates lower than 20% in Piantone di Mogliano, while in Piantone di Falerone the contamination level was 40.28% for the same treatment.

After the first three weeks of culture in the culture tubes, explants have been transferred on fresh ROM-based medium supplemented with 3 mg/L of BAP poured in micro boxes (Micropoli, IT) and checked weekly for the presence of possible fungal and bacterial contaminants. After 9 weeks of culture, the percentage of bud sprouting was nil in the cultivars Ascolana Tenera, 0.94% for Maurino, 1.38% for Piantone di Falerone and 11.85% for Piantone di Mogliano. Unfortunately, after 18 weeks of culture, only Piantone di Mogliano shoots were able to develop and proliferate in the selected ROM culture medium, and these explants have been used for the execution of the proliferation trial.

3.2. Proliferation

To determine efficient culture media for proliferation (multiplication), two different sets of experiments with varying types and combinations of plant growth regulators (PGR) were designed (see materials and methods). These proliferation media were employed for the proliferation of the olive cultivar “Piantone di Mogliano”. Data for each experiment were collected every 3 weeks, and the experiments were replicated three times, considering a sample composed by three microboxes.

3.2.1 Proliferation of cultivar Piantone di Mogliano from first set of plant growth regulators

The experimental trial has been set up in microboxes containing three explants for each container, for a total of 10 microboxes for each culture media used. In the presence of the control condition, characterized by the ROM medium without PGRs (P5: Proliferation medium 5), the other culture media had different cytokinin concentration: 0 mg/L of BAP + 3 mg/L of Zeatin (P1), 1 mg/L of BAP and 2 mg/L of Zeatin (P2), 2 mg/L of BAP and 1 mg/L of Zeatin (P3), 3 mg/L of BAP and 0.5 mg/L of Zeatin (P4) (Tables 14). The number of

healthy explants and the number of necrotic explants has been recorded every 3 weeks, transferring the explant on fresh newly prepared media, for a total of three sub-culture and 9 weeks of culture for the proliferation experiments. At the first sub-culture (3 weeks), the highest number of healthy proliferated explants has been observed in proliferation media 2 and 4 (Table 14). At the second sub-culture (6 weeks), the majority of healthy explants has been observed in proliferation media 1 and 4 (Table 15), and at the third sub-culture (9 weeks), the proliferation media 4 and 5 recorded the highest number of healthy explants (Table 16). These findings indicate that proliferation medium 4 yielded the best results for obtaining a healthy explant.

Table 14. Proliferation results at the first sub-culture (3 weeks) for the olive cultivar "Piantone di Mogliano".

Culture medium number	**BAP (mg/L)**	**Zeatin (mg/L)**	**starting explant (no)**	**Healthy Explant (no)**	**Explant Contaminated by bacteria (no)**	**Necrotic Explant (no)**
1	0	3	30	11	15	4
2	1	2	30	16	3	11
3	2	1	33	0	26	4
4	3	0.5	30	16	3	11
5	0	0	30	6	6	18

Table 15. Proliferation results at the second sub-culture (6 weeks) for the olive cultivar "Piantone di Mogliano".

Culture medium number	**BAP (mg/L)**	**Zeatin (mg/L)**	**Starting Explant (no)**	**Healthy Explant (no)**	**Explant Contaminated by bacteria (no)**	**Necrotic Explant (no)**
1	0	3	33	14	15	4
2	1	2	30	0	26	4
3	2	1	33	3	26	4
4	3	0.5	30	16	3	11
5	0	0	30	6	6	18

Table 16. Proliferation results at the third sub-culture (9 weeks) for the olive cultivar "Piantone di Mogliano"

Culture medium number	BAP (mg/L)	Zeatin (mg/L)	Starting Explant (no)	Healthy Explant (no)	Explant Contaminated by bacteria (no)	Necrotic Explant (no)
1	0	3	33	3	15	15
2	1	2	30	0	26	4
3	2	1	32	0	26	6
4	3	0.5	30	7	3	20
5	0	0	30	6	6	18

During the *in vitro* proliferation trial, differences between proliferation media have been detected in terms of bacterial contamination (Figure 29). At the first sub-culture, the highest percentage of bacterial contamination have been ascertained on proliferation medium 3 (P3), followed by P1, while the lowest contaminations have been noted on P2 and P4. At the second sub-culture, the highest percentage of bacterial contamination was associated to P2 and P3, and the lowest was related to P4 and P5. At the third sub-culture, the highest percentage of bacterial contamination was associated with P2 and P3, while the lowest was observed in P4. These results indicate that P2 was more infected by bacterial contamination, whereas P4 remained the least affected by bacterial contamination. These differences observed in the five-proliferation medium tested can be attributable to pre-existing contamination on plant material or newly contamination occurred during explant preparation.

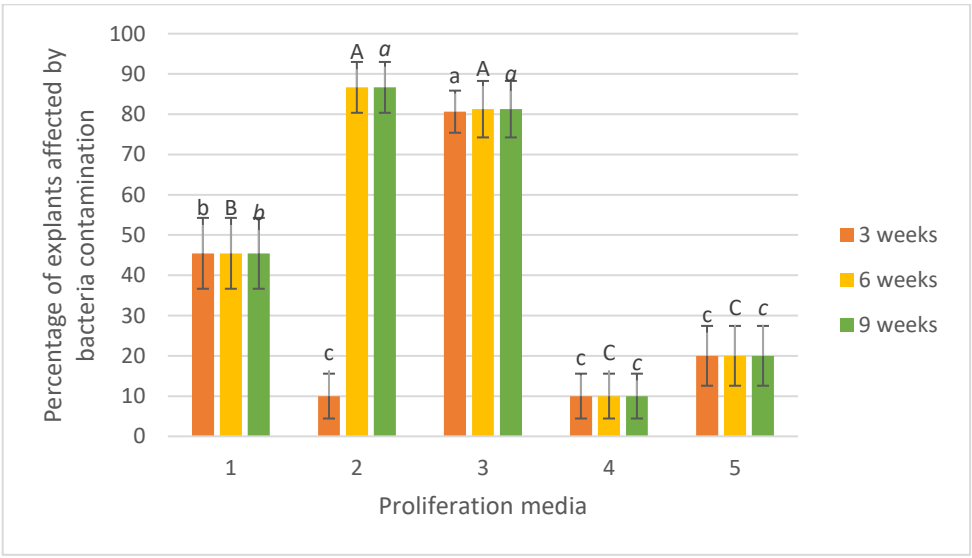

Figure 29: Comparison of bacterial contamination levels in the olive cultivar "Piantone di Mogliano" across five different proliferation media. Small letters represent differences in the percentage of bacterial contaminated explants between the different proliferation media at 3 weeks, capital letters represent the differences at 6 weeks, and small letter in italics at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 30$).

Concerning the percentage of necrosis, in the first and second sub-culture, the highest necrosis percentage have been associated to P5, followed by P4 and P2, while the lowest necrosis was related to P1 and P3. In the third sub-culture, the highest percentage of necrosis have been founded on P4, followed by P5 and P1, while the lowest necrosis level has been observed on P3. The results indicate that the highest levels of necrosis occurred in P4 and P5, while the lowest occurred in P3 (Figure 30).

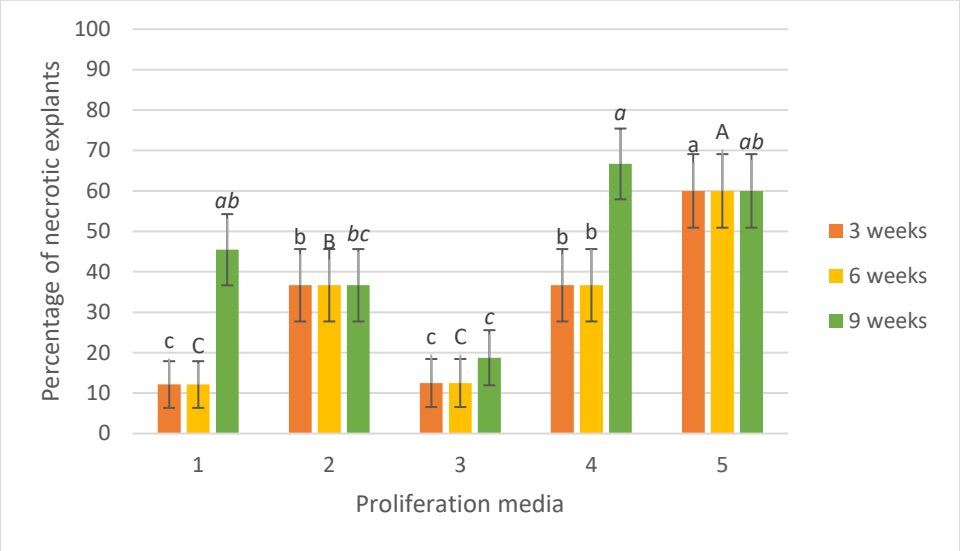

Figure 30: Comparison of necrosis levels in the olive cultivar "Piantone di Mogliano" across five different proliferation media. Small letters represent differences in the percentage of necrotic explants between the different proliferation media at 3 weeks, capital letters represent the differences at 6 weeks, and small letter in italics at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 30$).

The proliferating shoots have been counted for each explant cultured on the different proliferation media after each sub-culture, and the results showed that the highest average number of shoots have been founded on P1, followed by P3, while the lowest number of proliferating shoots have been observed on P5 (Figure 31). Therefore P1, P3, and P4 at 3 weeks of culture seems to be the best proliferation media, while at 9 weeks of culture only P1 and P3 were statistically better than the control medium (P5).

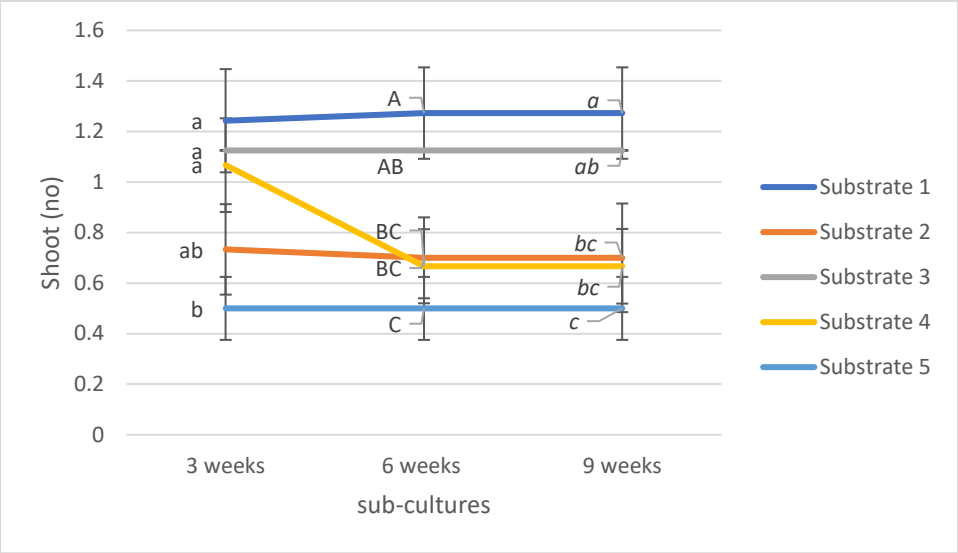

Figure 31: Average number of shoots in the olive cultivar "Piantone di Mogliano" across five different proliferation media over three sub-cultures. Small letters represent differences in the average number of shoots between the different proliferation media at 3 weeks, capital letters represent the differences at 6 weeks, and small letter in italics at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 30$).

The shoots primordia that appeared as green translucid buds at the base of the nodal cuttings (Figure 32) has been counted after the second and third sub-culture (Figure 32). At the second sub-culture, the highest number of primordial shoots has been counted on P3, followed by P1, P2, and P4 (Figure 32); however, the statistical analysis did not show any significant difference between them. At the third sub-culture, the highest number of primordial shoots has been counted on P1, followed by P3, P2, and P4 (Figure 32); however, the statistical analysis did not show any significant difference between them (Figure 15).

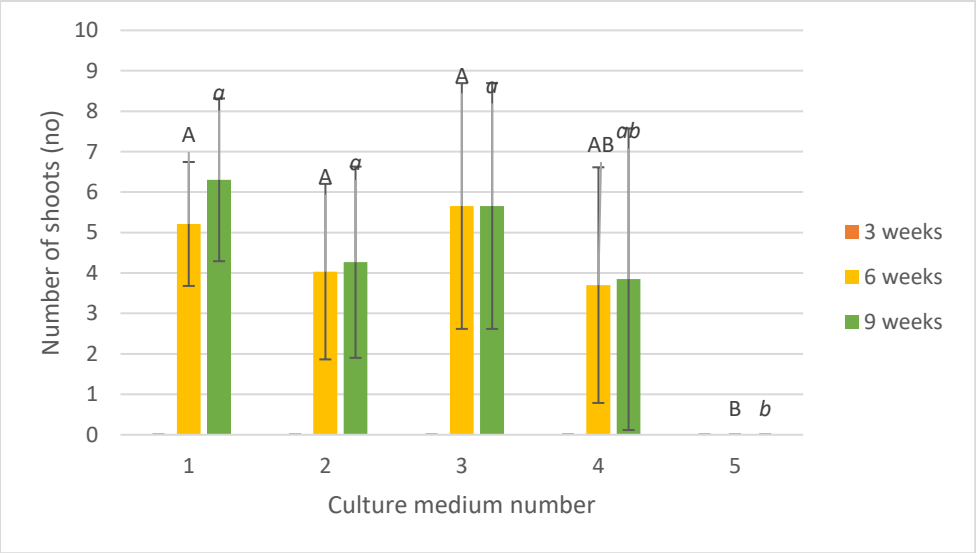

Figure 32: Number of shoots primordia in the olive cultivar "Piantone di Mogliano" across five different proliferation media over three sub-cultures. Capital letters represent differences in the average number of shoot primordia between the different proliferation media at 6 weeks, small letters in italics represent the differences at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 30$).

At each sub-culture the plant material cultured in the absence of PGRs slowly necrotized (Figure 33 E; Figure 35 D), due to the absence of cytokinins able to stimulate the proliferation of meristematic cells, while in all the other proliferation media, callogenesis (Figure 33 A, C; Figure 34 B-C; Figure 35 A-B) and the development of lateral sprouts (Figure 33 B; Figure 34 A, D; Figure 35 C) occurred, without any visible difference among the various proliferation media tested.

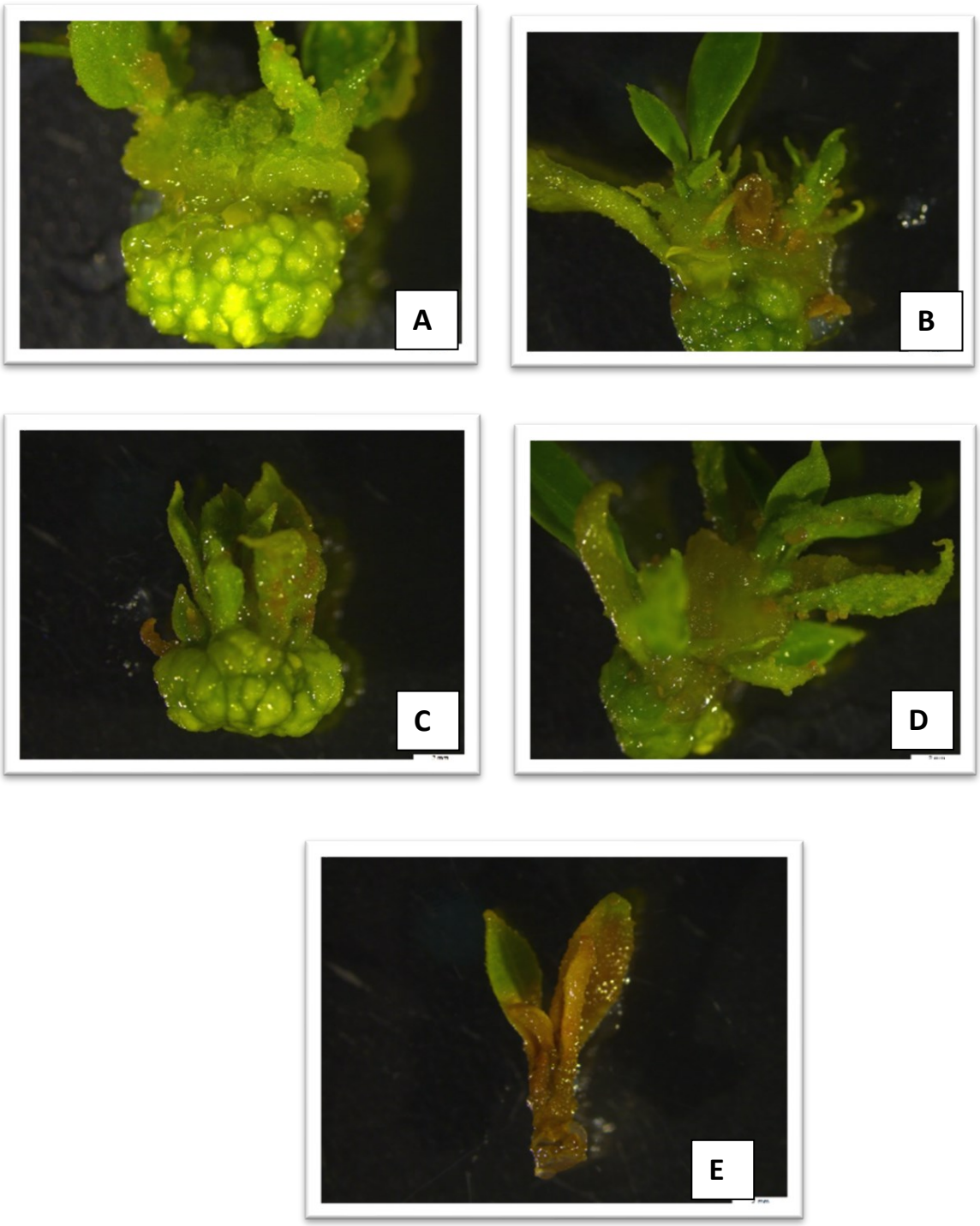

Figure 33: proliferated olive Cultivar “Piantone di Mogliano” after the first sub-culture (3weeks); P1(A); P2(B); P3(C); P4(D); P5(E).

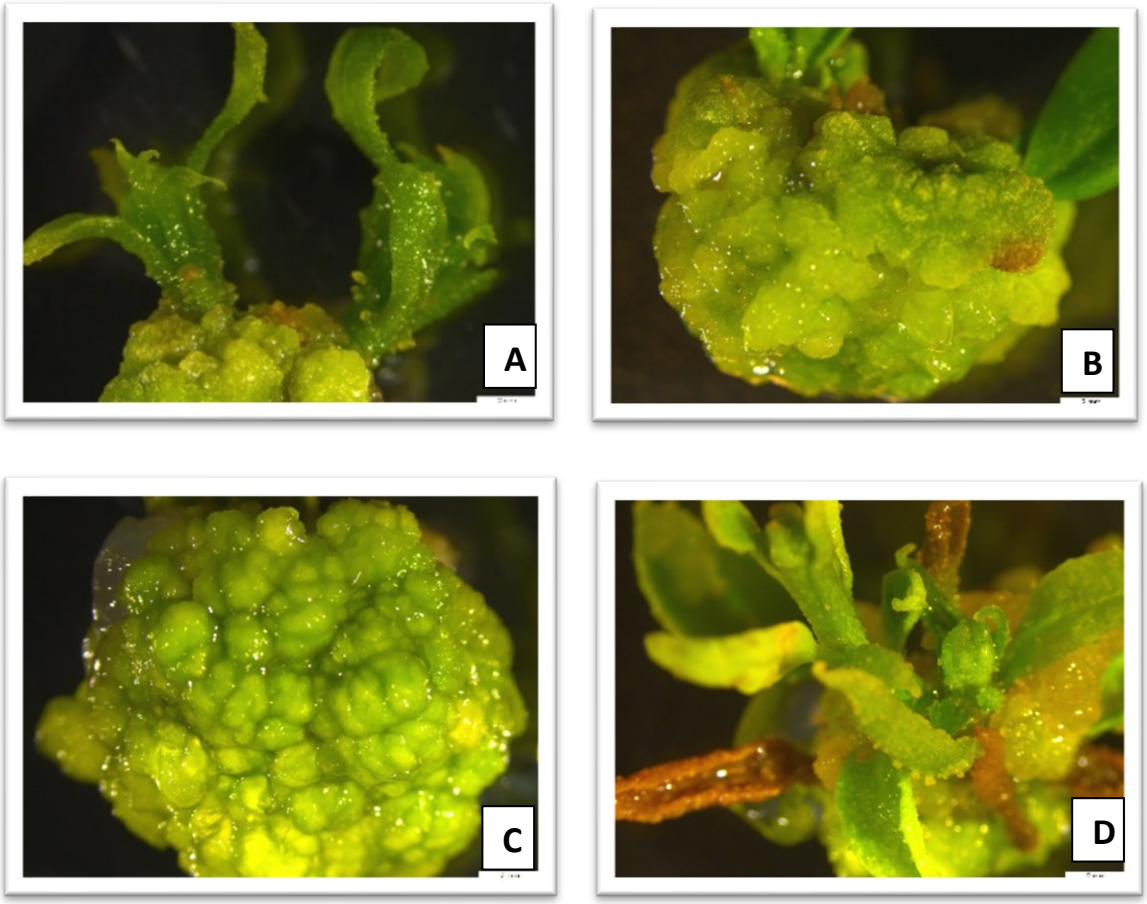

Figure 34: Proliferated olive cultivar “Piantone di Mogliano” after the second sub-culture (6 weeks); P1(A); P2(B); P3(C); P4(D).

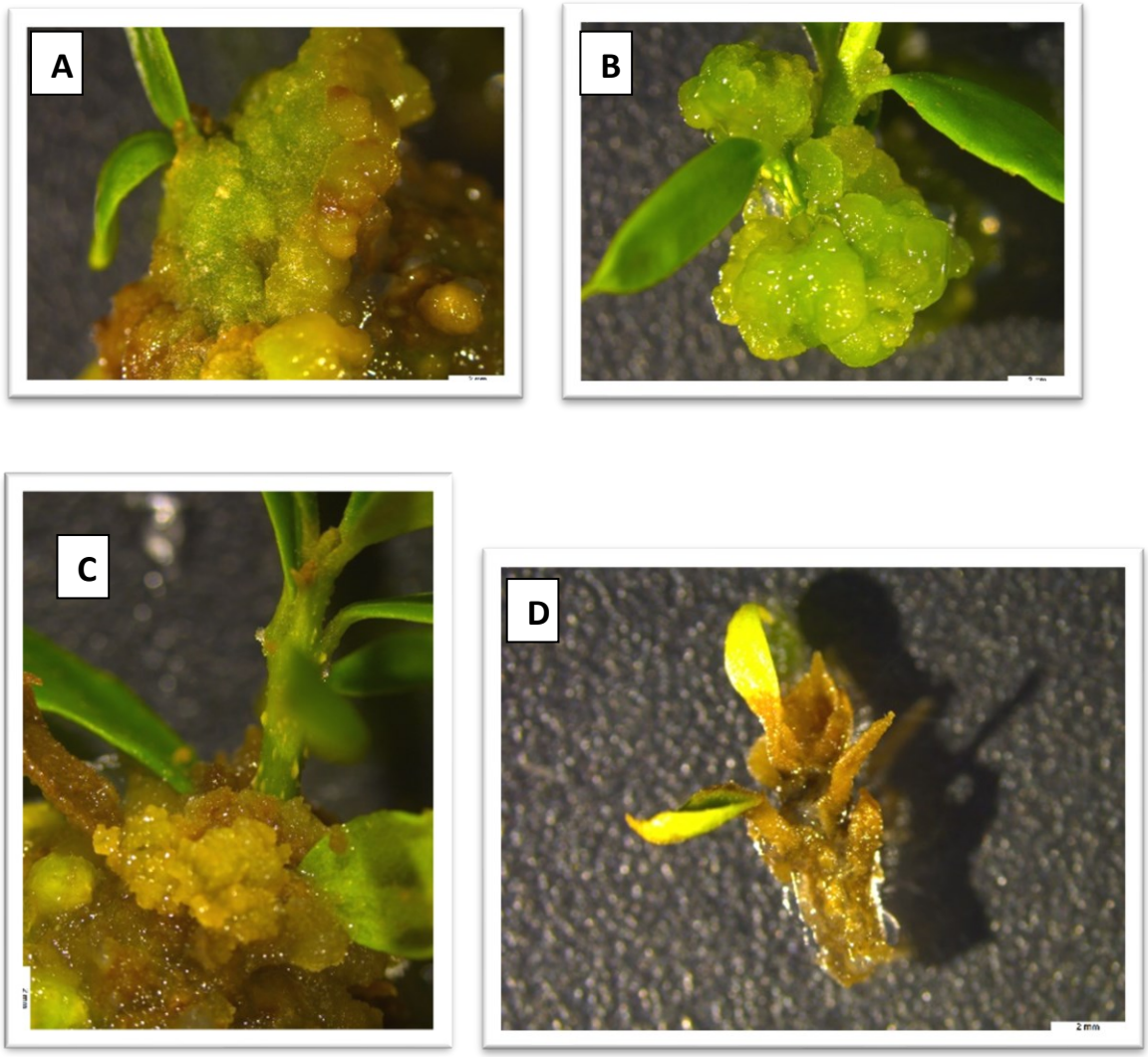

Figure 35: Proliferated olive cultivar “Piantone di Mogliano” after the third sub-culture (9 weeks); P1(A); P3(B); P4(C); P5(D).

The callogenesis that appeared at the base of each cutting, has been considered in the results. Figure 36 demonstrate the percentage of callogenesis observed at 3, 6, and 9 weeks of culture at the base of each cutting. The lowest percentage was related to the proliferation media P5 followed by P2. The highest number of calli were related to proliferation media P3 and P1.

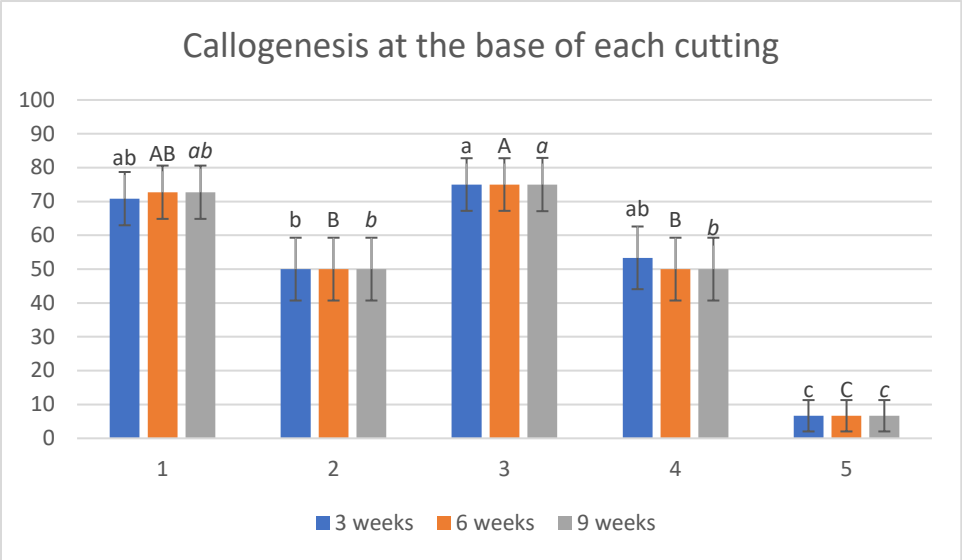

Figure 36: Callogenesis at the base of each cutting in the olive cultivar "Piantone di Mogliano" across five different proliferation media over three sub-cultures. Capital letters represent differences in the average number of shoot primordia between the different proliferation media at 6 weeks, small letters in italics represent the differences at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 30$).

Additionally, callus formed in each subculture (Figure 33 A-D; Figure 34 A-D; Figure 35 A-C) was cut and cultivated in subsequent subcultures to monitor whether they could develop into embryonic primordia, but they did not form any embryonic primordia.

Based on our results, necrosis and browning at the shoot base were observed after 3 weeks in subculture on P5 due to the absence of any plant growth regulators (ZEATIN, BAP). In fact, necrosis was observed in all explants and in all the sub-cultures occurred on P5 (Figure 33 E; Figure 35 D).

3.2.2 Concluding Proliferation Outcomes from first set of plant growth regulators

The results presented in Tables 14 to 16 and Figures 29 to 36 offer a comprehensive view of the performance of different proliferation media in the cultivation of olive explants. Examining the initiation phase, the data reveals noteworthy trends in the development of healthy proliferated shoots over three sub-cultures. At the end of the first sub-culture at 3 weeks, proliferation media 2 and 4 demonstrated to maintain the highest numbers of healthy explants, while at the second sub-culture (6-weeks), the highest number of healthy explants have been reported in proliferation media 1 and 4. Surprisingly, at the end of the third sub-culture (9 weeks), proliferation media 4 and 5 exhibited the highest numbers of healthy samples, suggesting proliferation medium 4 as the most sterile, avoiding bacterial and fungal contaminations.

Bacterial contamination was a significant factor influencing the efficacy of the *in vitro* proliferation trial, as highlighted in Figure 29. Proliferation medium 3 showed the highest bacterial contamination in the first sub-culture, with P2 and P4 exhibiting the least contamination. At the second sub-culture, proliferation media 2 and 3 were associated with the highest contamination, while proliferation media 4 and 5 displayed the lowest. Remarkably, a similar pattern emerged in the third sub-culture, emphasizing the major bacteria contaminants in the proliferation media 2 and 3, and the minimum level of bacterial contamination founded on proliferation media 4 and 5.

Necrosis percentages, discussed in the context of proliferation media 1, 2, 3, 4, and 5, further delineate culture medium responses. In the first and second sub-culture, P5 exhibited the highest necrosis percentage, followed by proliferation media 4 and 2, while P1 and P3 showed the lowest levels. The third sub-culture highlighted P4 and P5 as the proliferation media with the highest necrosis, and P3 with the lowest. This implies that proliferation media 4 and 5 were prone to have higher necrosis levels, contrasting with the resilience observed in P3.

Analyzing shoot development (Figure 31), P1 consistently outperformed others, yielding the highest average number of shoots after each sub-culture, followed by proliferation media 3 that was not statistically different to P1. Conversely, proliferation medium 5 consistently displayed the lowest shoot development. Positive shoot development could be correlated with the presence of Zeatin, whereas we have 3 and 1 mg/L of it in P1 and P3, respectively. However, there is no Zeatin in P5. Findings by Titouh et al. (2022) also confirmed our results, demonstrating a positive effect of zeatin on the *in vitro* shoot development of the Algerian olive cultivar “Chemlal”.

Focusing on shoots primordia (Figure 32), the statistical analysis did not reveal significant differences in their numbers among proliferation media during both the second and third sub-culture. Proliferation medium 3 recorded the highest number in the second sub-culture, while proliferation medium 1 exhibited the highest number of shoot primordia in the third sub-culture. Despite differences in absolute numbers have been noted, the lack of statistical significance suggests comparable performance among these culture media in supporting the development of shoots primordia.

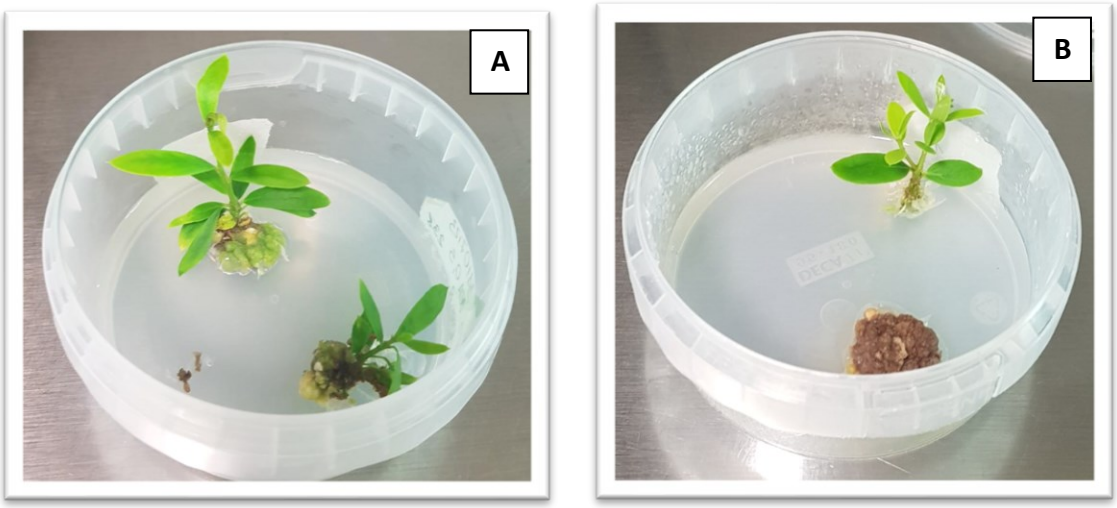

Figure 37: Proliferated shoots belonging to the olive cultivar “Piantone di Mogliano; on proliferation medium 4 (A), and proliferation medium 2 (B).

3.2.3 Proliferation of cultivar Piantone di Mogliano from second set of plant growth regulator

Based on the plant material availability, the experimental trial has been set up in glass pots (500 mL of total capacity) containing two explants for each container, for a total of 5 glass pots for each culture media used. The specific proliferation media, based on ROM basal salts and vitamins had different cytokinin concentration, almost exclusively using BAP as cytokinin”s source: 3 mg/L of BAP (P6), 4 mg/L of BAP (P7), 5 mg/L of BAP (P8) (Tables 8). The number of healthy explants and the number of necrotic explants has been recorded every 3 weeks, for a total of 9 weeks of culture for the proliferation experiments. Tables 17 to 19 present the number of starting explants, along with the associated, as well as details concerning healthy, contaminated, and necrotic samples for each treatment after proliferation. At the first sub-culture (3 weeks), the highest percentage of healthy proliferated plants belonged to proliferation media 6 and 7 (Table 17). In the second sub-culture (6 weeks), P8 was the most effective medium in terms of highly healthier proliferative explants (Table 18), and at the third sub-culture (9 weeks), the proliferation medium 8 confirmed to be the treatment having the healthier explants (Table 19).

Table 17. Proliferation results of the first sub-culture (3 weeks) for the olive cultivar "Piantone di Mogliano"

Proliferation medium number	BAP (mg/L)	Zeatin (mg/L)	Starting Explant (no)	Healthy Explant (no)	Explant Contaminated by bacteria (no)	Necrotic Explant (no)
6	3	0	10	7	3	0
7	4	0	11	10	0	0
8	5	0	10	5	0	5

Table 18. Proliferation results of the second replication (6 weeks) for the olive cultivar "Piantone di Mogliano"

Proliferation medium number	BAP (mg/L)	Zeatin (mg/L)	Starting Explant (no)	Healthy Explant (no)	Explant Contaminated by bacteria (no)	Necrotic Explant (no)
6	3	0	10	7	3	0
7	4	0	12	10	0	2
8	5	0	9	9	0	0

Table 19. Proliferation results of the third replication (9 weeks) for the olive cultivar "Piantone di Mogliano"

Proliferation medium number	BAP (mg/L)	Zeatin (mg/L)	Starting Explant (no)	Healthy Explant (no)	Explant Contaminated by bacteria (no)	Necrotic Explant (no)
6	3	0	10	3	1	6
7	4	0	10	9	0	1
8	5	0	9	9	0	0

Differences between proliferation media in terms of bacterial contamination have been found (Figure 38). In all sub-cultures, the highest percentage of bacterial contamination was associated to proliferation medium 6, while the proliferation media 7 and 8 did not show any bacterial contamination. The results indicate that there was a bacterial contamination problem on proliferation medium 6 (about 10%), probably due to a pre-

existing plant contamination or to an environmental contamination occurred during plant material propagation. Given the low level of contamination, no relevant differences were detected among P6, P7, and P8.

Concerning the percentage of necrosis, in the first sub-culture, just P8 showed necrosis (50%). In the second sub-culture the sole proliferation medium 7 showed necrosis (15%), while in the third sub-culture proliferation media 6 (10%) and 7 (15%) showed necrosis. In the third sub-culture, the highest percentage of necrosis was associated with proliferation medium 6.

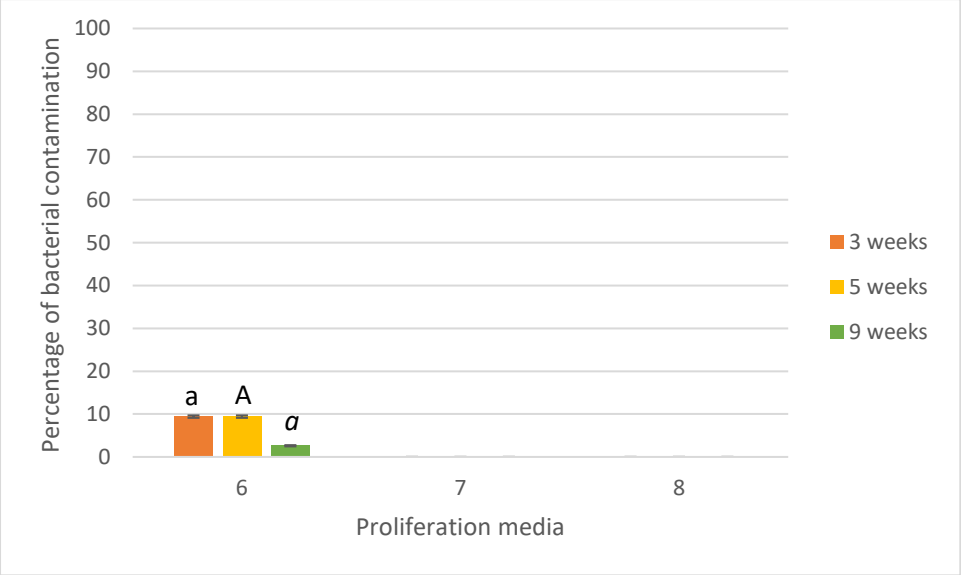

Figure 38: Comparison of bacterial contamination levels in the olive cultivar "Piantone di Mogliano "cultured on three different proliferation media (P6, P7, P8). Small letters represent differences in the percentage of bacterial contaminated explants between the different proliferation media at 3 weeks, capital letters represent the differences at 6 weeks, and mall letter in italics at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 10$).

The number of shoots proliferated in each medium was counted after each sub-culture, and the results showed that the highest average number of shoots was counted in culture medium 8 at the second and third sub-culture (Figure 39).

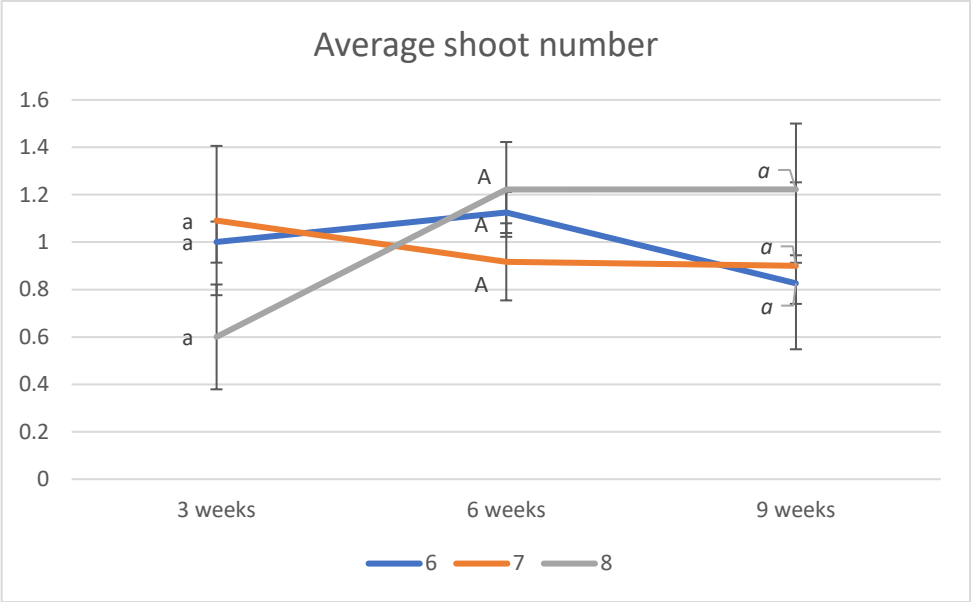

Figure 39: Average number of shoots in the olive cultivar "Piantone di Mogliano" across three different proliferation media over three sub-cultures. Small letters represent differences in the average number of shoots between the different proliferation media at 3 weeks, capital letters represent the differences at 6 weeks, and small letter in italics at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 10$).

The number of nodes in each proliferating shoot was counted after each sub-culture. The results indicated that the highest average number of nodes belonged to culture medium 8 during the third sub-culture (Figure 40). The least effective was proliferation medium 7, however, statistical analysis did not reveal any significant differences among the media.

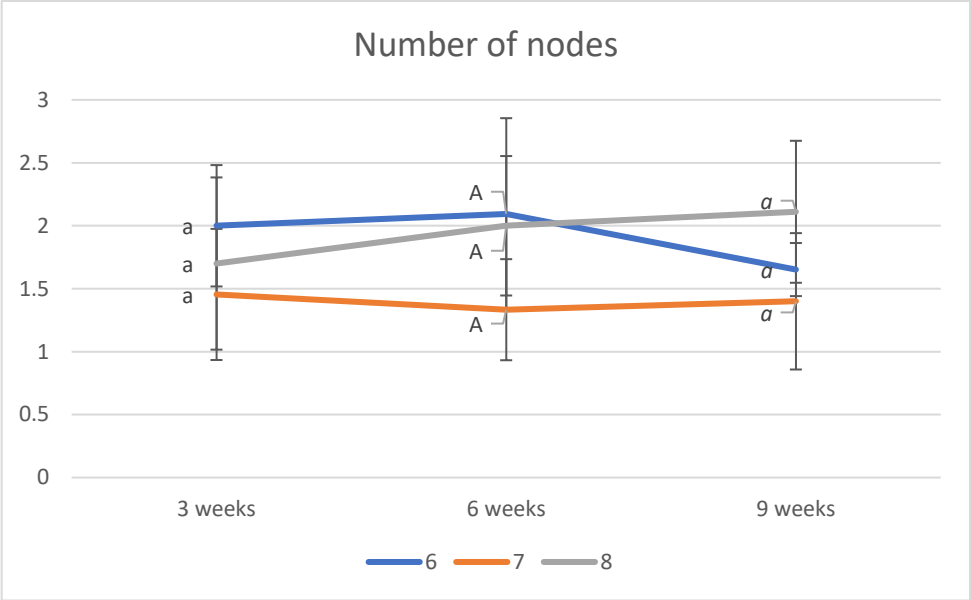

Figure 40: Average number of nodes in the olive cultivar "Piantone di Mogliano" across three different proliferation media over three sub-cultures. Small letters represent differences in the average number of shoots between the different proliferation media at 3 weeks, capital letters represent the differences at 6 weeks, and small letter in italics at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 10$).

Concerning the average number of lateral shoots (Figure 41), P6 exhibited the highest average. However, the statistical analysis did not detect any notable distinctions among the various media.

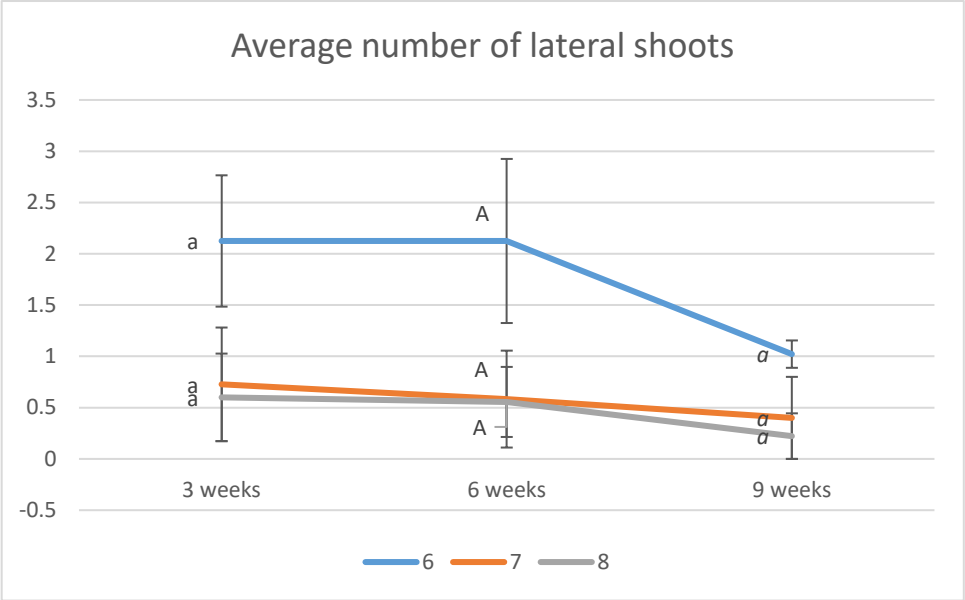

Figure 41: Average number of lateral shoots in the olive cultivar "Piantone di Mogliano" across three different proliferation media over three sub-cultures. Small letters represent differences in the average number of shoots between the different proliferation media at 3 weeks, capital letters represent the differences at 6 weeks, and small letter in italics at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 10$).

During the second proliferation trial of the olive cultivar "Piantone di Mogliano", the majority of the explants formed green callus at the base of almost each nodal cutting, in the portion completely immersed in the proliferation media (Figure 42 A). The emergence of new lateral shoots occurred especially on P6 at the second and third sub-culture (Figure 42 B-C).

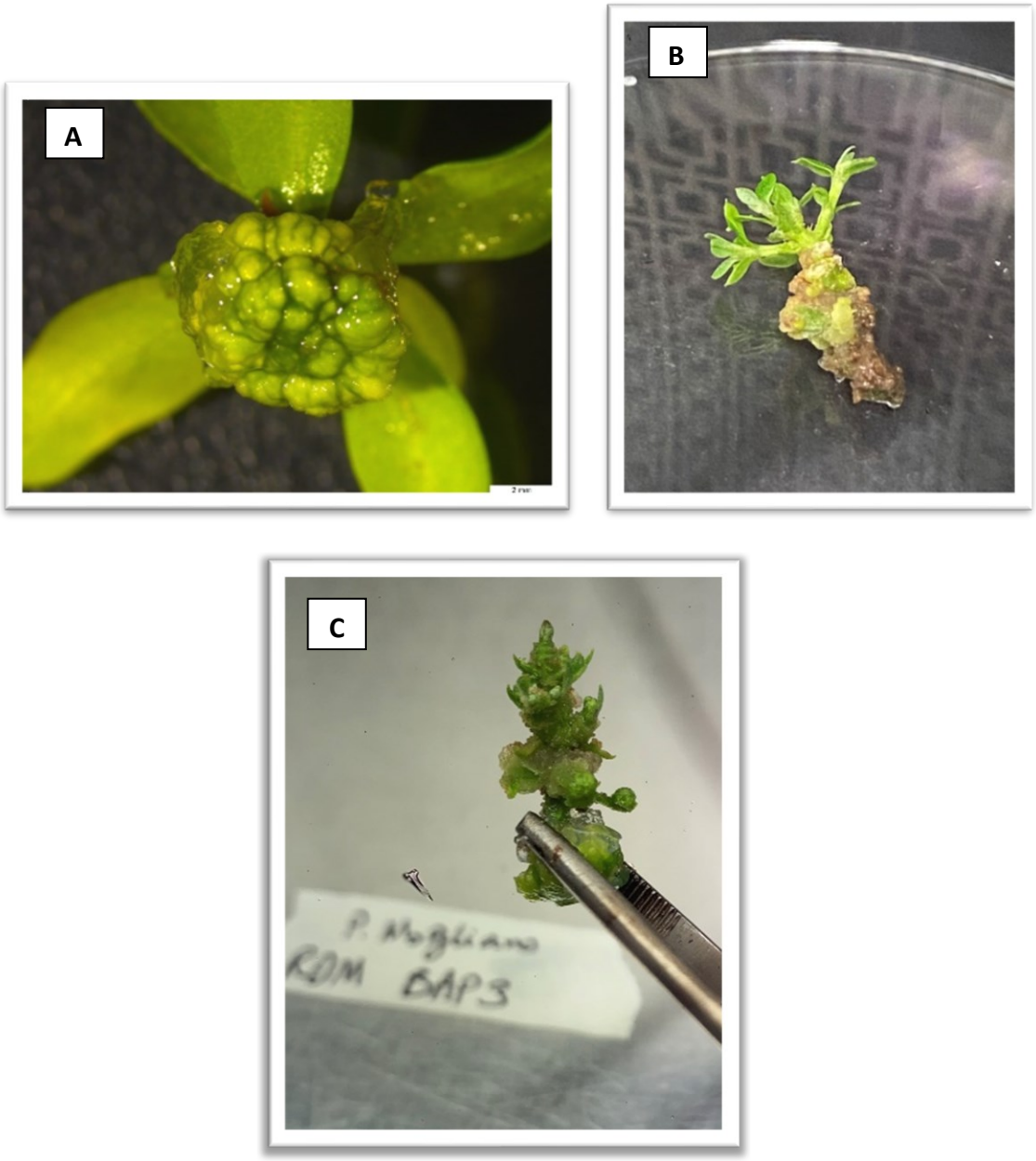

Figure 42: *in vitro* proliferation of Olive cultivar "Piantone di Mogliano" for the proliferation medium 6; first sub-culture (3 weeks) (A); Second sub-culture (6 weeks) (B); third sub-culture (9 weeks) (C).

The callogenesis that appeared at the base of each cutting, has been considered as an additional parameter Figure 43 demonstrate the percentage of callogenesis occurred on the various proliferation media at each sub-culture. The lowest percentage is related to the proliferation media P8 at the 9 weeks.

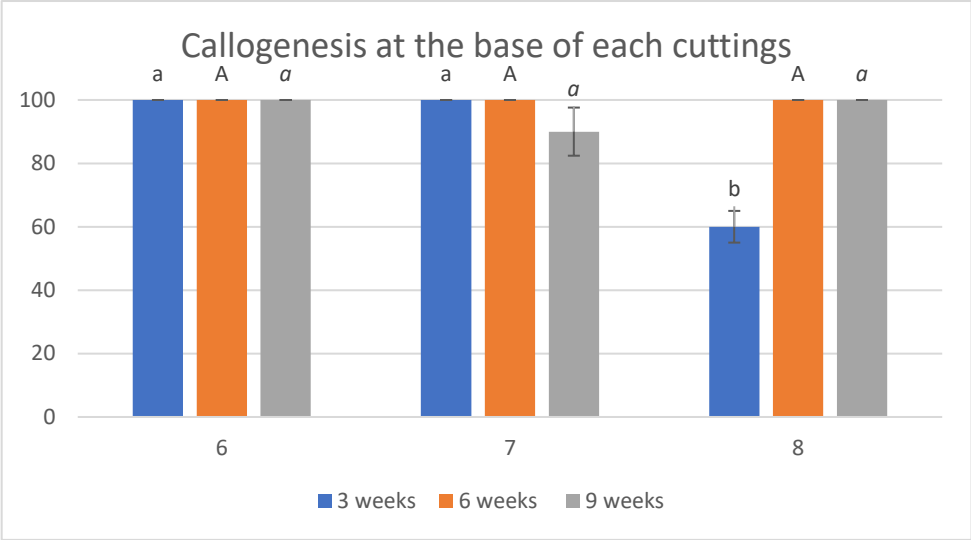

Figure 43: Callogenesis at the base of each cutting in the olive cultivar "Piantone di Mogliano" across three different proliferation media over three sub-cultures. Capital letters represent differences in the average number of shoot primordia between the different proliferation media at 6 weeks, small letters in italics represent the differences at 9 weeks. Means with different letters are significantly different according to Duncan test ($p < 0.05$) ± SE ($n = 30$).

Furthermore, callus formed in each subculture was excised and transferred to subsequent subcultures to assess its potential to develop into embryonic primordia. However, no embryonic primordia were observed. The formation of callus in the substrate with second set of PGR was less than that with first set of PGR, which could be a result of the presence of Zeatin in the first set of PGR.

3.2.4 Concluding Proliferation Outcomes from second set of plant growth regulators

The results presented in Tables 17 to 19 and Figures 38 to 43 offer a comprehensive view of the performance of different proliferation media in the *in vitro* cultivation of olive explants. Examining the initiation phase, the data reveals noteworthy trends in the development of healthy proliferated samples over three sub-cultures. During the first sub-culture at 3 weeks, proliferation media 6 and 7 demonstrated to maintain the highest numbers of healthy explants. Surprisingly, in the second sub-culture (6-week) and third sub-culture (9-week), proliferation medium 8 had the healthiest explants.

Bacterial contamination was a significant factor influencing the efficacy of the experimental trial, as highlighted in Figure 37. Proliferation medium 6 showed the highest bacterial contamination in all sub-culture, while proliferation media 7 and 8 did not show any bacterial contamination during all sub-cultures.

Necrosis percentages, discussed in the context of proliferation media 6, 7, and 8, further delineate culture medium responses. In the first sub-culture, proliferation medium 8 exhibited the highest percentage of necrosis;

in the second sub-culture, P7 showed the highest percentage, and in the third sub-culture, P6 exhibited the highest percentage of necrosis. This implies that there was no significant trend in identifying the most sensitive proliferation medium to necrosis among P6 to P8.

Upon analyzing shoot development (Figure 38), proliferation medium 8 consistently outperformed others, producing the highest average number of shoots after the second and third sub-cultures. It could be attributed to the presence of BAP, with the highest concentration observed in P8 at 5 mg/L. Finding by Titouh et al. (2022) also validate our results, demonstrating the positive impact of Benzylaminopyrine (BAP) on *in vitro* shoot development in the Algerian olive cultivar 'Chemlal.' In their study, the addition of BAP to two different substrates resulted in better shoot development compared to the control (without hormone), showing significant statistical differences. Although P8 seems to stimulate the enhancement of the number of proliferating shoots, P6 consistently increased the number of lateral shoots sprouted on the proliferating shoots, leading to the obtainment and the isolation of an increased number of *in vitro* shoots.

CONCLUSIONS

In conclusion, this study focused on the development of an efficient *in vitro* sterilization and micropropagation protocols of four olive cultivars: “Ascolana Tenera”, “Piantone di Mogliano”, “Piantone di Falerone”, and “Maurino”. The experimental trials were specifically designed to optimize *in vitro* sterilization and proliferation protocols, aiming to identify the most effective combination of exposure time and sterilizing agent concentration to maximize the production of healthy plants adaptable to *in vitro* conditions. Additionally, the study aimed to define a suitable combination and concentration of plant growth regulator to enhance in vitro shoot proliferation.

The results demonstrated that the sterilizing agent sodium hypochlorite is more effective than hydrogen peroxide (H_2O_2 with concentration of 3%). The investigation revealed that sodium hypochlorite treatments, specifically treatment 5 (15% for 15 minutes) and treatment 6 (15% for 10 minutes), showed the highest efficiency in sterilization across all cultivars. Furthermore, the cultivar “Maurino” exhibited the highest resistance to high concentrations of sodium hypochlorite, followed by the cultivars “Piantone di Falerone and “Piantone di Mogliano”.

During the proliferation phase, the results indicated distinct trends in the performance of different proliferation media. In the first set of plant growth regulators (consisting of BAP and Zeatin), proliferation medium 4 (with 3 mg/L BAP and 0.5 mg/L Zeatin) exhibited the highest efficiency in supporting healthy explant development. However, media 4 and 5 showed a higher susceptibility to necrosis. In the second set of plant growth regulators (consisting of BAP without Zeatin), proliferation medium 8 consistently outperformed others in supporting shoot development. The high concentration of BAP in P8 (5 mg/L) was identified as a contributing factor to its superior performance. The positive effects of Zeatin and BAP on the in vitro cultivation of olive explants and shoot development in olive cultivars were consistent with other research, such as the findings by Titouh et al. (2022). Regarding the formation of callus, Zeatin was observed as an important parameter that stimulated the existence of callus. However, the potential for its development into embryonic primordia was not observed.

During the experiment with the first and second set of PGRs, it was observed that there was no difference between the average shoot numbers at 6 and 9 weeks. Therefore, a longer time will not improve it, and the material will just enter senescence.

Overall, the study provides valuable insights into the development of efficient micropropagation protocols for olive cultivars, contributing to the advancement of in vitro cultivation techniques for this economically important species. Future research could further explore and refine these protocols, considering the specific needs and characteristics of different olive cultivars.

BIBLIOGRAPHY

Abuzayed, M., Frary, A., & Doganlar, S. (2018). Genetic diversity of some Palestinian and Turkish olive (Olea europaea L.) germplasm determined with SSR markers. *IUG Journal of Natural Studies*, *26*(1).

Ahmad, N., Khan, N., Ali, F., Rahman, K. U., Bahadar, S., Khan, M. I., ... & Bahadar, R. (2016). Comparison of Different Parameters for the In Vitro Propagation of Various Cultivars of Olive (Olea Europaea L.). *International Journal of Basic Medical Sciences and Pharmacy (IJBMSP)*, *6*(1).

Bao, Z. H., Ma, Y. F., Liu, J. F., Wang, K. J., Zhang, P. F., Ni, D. X., & Yang, W. Q. (1980). The induction of plantlets from the hypocotyl of Olea europaea in vitro. *Acta Botanica Sinica*, *22*(1), 96-97

Barranco, D. (1999) Variedades y patrones. In: D. Barranco, R. Fernandez-Escobar and L. Rallo ´ (Eds.) El Cultivo del Olivo, 3rd Ed. Ediciones Mundi-Prensa, Madrid, pp. 63–89

Barranco, D., Navero, D. B., & Romero, L. R. (2008). *El cultivo del olivo*. Mundi-Prensa.

Bartolini, G., Prevost, G., Messeri, C., & Carignani, G. (1998). Olive germplasm: cultivars and world-wide collections

Bayraktar, M., Hayta-Smedley, S., Unal, S., Varol, N., & Gurel, A. (2020). Micropropagation and prevention of hyperhydricity in olive (Olea europaea L.) cultivar "Gemlik". *South African Journal of Botany*, *128*, 264-273

Beasley DM, Schep LJ, Slaughter RJ, Temple WA, Michel JM. Full recovery from a potentially lethal dose of mercuric chloride. J Med Toxicol. 2014 Mar;10(1):40-4. doi: 10.1007/s13181-013-0311-1. PMID: 23760886; PMCID: PMC3951629.

Boskou, D., Blekas, G., & Tsimidou, M. (2006). Olive oil composition. In *Olive oil* (pp. 41-72). AOCS press

Cecchi, L., Migliorini, M., Cherubini, C., Giusti, M., Zanoni, B., Innocenti, M., & Mulinacci, N. (2013). Phenolic profiles, oil amount and sugar content during olive ripening of three typical Tuscan cultivars to detect the best harvesting time for oil production. *Food research international*, *54*(2), 1876-1884.

Darwesh, O. M., Hassan, S. A., & Abdallatif, A. M. (2022). Improve In vitro Multiplication of Olive Shoots Using Environmental-Safe Chitosan, Selenium, and Silver Nanostructures

de Almeida Cançado, G. M., Setotaw, T. A., & Ferreira, J. L. (2013). Applications of biotechnology in olive. *African Journal of Biotechnology*, *12*(8)

DE SIMONE, D. S. M., & CARUSO, C. M. P. T. (2014). INDAGINE SULLA DIVERSITÀ INTRA-VARIETALE NELLE PRINCIPALI CULTIVAR DI OLIVO (Olea europaea L.) SICILIANE.

Diez, C. M., Trujillo, I., Martinez-Urdiroz, N., Barranco, D., Rallo, L., Marfil, P., & Gaut, B. S. (2015). Olive domestication and diversification in the Mediterranean Basin. *New Phytologist*, *206*(1), 436-447

Fabbri A (2006) Olive propagation: new challenges and scienti fi c research. In: Proc 2nd Int Seminar OLIVEBIOTEQ 2006, vol. I. Marsala, Mazara del Vallo, Italy, pp. 411–421

Fabbri, A., Bartolini, G., Lambardi, M. and Kailis, S. (2004) Olive Propagation Manual. CSIRO Publ., Australia, pp. 130.

Fabbri, A., Lambardi, M., & Ozden-Tokatli, Y. (2009). Olive breeding. *Breeding plantation tree crops: tropical species*, 423-465

Fernández, F. J., Ladux, J. L., Hammami, S. B., Rapoport, H. F., & Searles, P. S. (2018). Fruit, mesocarp, and endocarp responses to crop load and to different estimates of source: sink ratio in olive (cv. Arauco) at final harvest. *Scientia Horticulturae*, *234*, 49-57

Fernández, J.E., Moreno, F. and Martin-Aranda, J. (1990) Study of root dynamics of olive trees under drip irrigation and dry farming. Acta Horticulturae 286, 263–266.

Fraga, H., Pinto, J. G., Viola, F., & Santos, J. A. (2020). Climate change projections for olive yields in the Mediterranean Basin. *International Journal of Climatology*, *40*(2), 769-781.

Friedrich, W. L. (1980). Fossil plants from Weichselian interstadials, Santorini (Greece) II. *Thera and the Aegean World II. London: Thera and the Aegean World*, 109-128

Grigoriadou, K., Eleftheriou, E.P., Vasilakakis, M., 2007. Hidden hyperhydricity may be responsible for abnormal development and acclimatization problems of micropropagated olive plantlets: an anatomical leaf study. In: Santamaria, J.M., Desjardins, Y. (Eds.), II International Symposium On Acclimatization and Establishment of Micropropagated Plants. 748, Acta Horticulturae, pp. 103–106. https://doi. org/10.17660/ActaHortic.2007.748.10.

Grigoriadou, K., Vasilakakis, M., & Eleftheriou, E. P. (2002). In vitro propagation of the Greek olive cultivarChondrolia Chalkidikis'. Plant Cell, Tissue and Organ Culture, 71, 47-54.

Gucci, R., Lodolini, E. M., & Rapoport, H. F. (2009). Water deficit-induced changes in mesocarp cellular processes and the relationship between mesocarp and endocarp during olive fruit development. *Tree physiology*, *29*(12), 1575-1585

Hackett, W. P. (2011). Juvenility, maturation, and rejuvenation in woody plants. *Horticultural reviews*, *7*, 109-154

Hassan, S. A. M., & Zayed, N. S. (2018). Factor controlling micropropagation of fruit trees: a review. *Science International*, *6*(1), 1-10

Hatzopoulos, P., Banilas, G., Giannoulia, K., Gazis, F., Nikoloudakis, N., Milioni, D., & Haralampidis, K. (2002). Breeding, molecular markers and molecular biology of the olive tree. *European Journal of Lipid Science and Technology*, *104*(9-10), 574-586

(IOC, http://www.internationaloliveoil.org/)

Khosravi, A., Zucchini, M., Giorgi, V., Mancini, A., & Neri, D. (2021). Continuous monitoring of olive fruit growth by automatic extensimeter in response to vapor pressure deficit from pit hardening to harvest. *Horticulturae*, *7*(10), 349.

Kumar, R., Alamgir, V. Y., Pal, D., & Shukla, R. Micropropagation in Horticultural Crops

Lambardi M, Rugini E (2003) Micropropagation of olive (Olea europaea L.). In: Jain SM, Ishii K (eds) Micropropagation of woody trees and fruits. Kluwer, Dordrecht

Lambardi, M., Ozudogru, E.A., Roncasaglia, R., 2013. In vitro propagation of olive (Olea europaea L.) by nodal segmentation of elongated shoots. In: Lambardi, M., Ozudogru, E., Jain, S. (Eds.), Protocols For Micropropagation of Selected Economically-Important Horticultural plants: Methods in Molecular Biology (Methods and Protocols). 994, Humana Press, Totowa, NJ, pp. 33–44. https://doi.org/10.1007/ 978-1-62703-074-8_3

Martínez-Ballesta, M. C., Alcaraz-López, C., Muries, B., Mota-Cadenas, C., & Carvajal, M. (2010). Physiological aspects of rootstock–scion interactions. *Scientia Horticulturae*, *127*(2), 112-118

Milicevic, M., Zubrinic, K., Grbavac, I., & Obradovic, I. (2020). Application of deep learning architectures for accurate detection of olive tree flowering phenophase. *Remote Sensing*, *12*(13), 2120.

Moreno-Alías, I., León, L., de la Rosa, R., & Rapoport, H. F. (2009). Morphological and anatomical evaluation of adult and juvenile leaves of olive plants. *Trees*, *23*, 181-187

Palliotti, A., & Bongi, G. (1996). Freezing injury in the olive leaf and effects of mefluidide treatment. *Journal of Horticultural Science*, *71*(1), 57-63

Parri, S., Romi, M., Hoshika, Y., Giovannelli, A., Dias, M. C., Piritore, F. C., ... & Cantini, C. (2023). Morpho-Physiological Responses of Three Italian Olive Tree (Olea europaea L.) Cultivars to Drought Stress. *Horticulturae*, *9*(7), 830

Pasqualone, A., Montemurro, C., di Rienzo, V., Summo, C., Paradiso, V. M., & Caponio, F. (2016). Evolution and perspectives of cultivar identification and traceability from tree to oil and table olives by means of DNA markers. *Journal of the Science of Food and Agriculture*, *96*(11), 3642-3657

Pollastri, S. (2008). Biotechnological advances in olive (Olea europaea L.): a review. *Biotechnological Advances in Olive (Olea europaea L.)*, 1000-1006

Proietti, P., Nasini, L., & Ilarioni, L. (2012). Photosynthetic behavior of Spanish Arbequina and Italian Maurino olive (Olea europaea L.) cultivars under super-intensive grove conditions. *Photosynthetica*, *50*, 239-246

Regni, L., Del Buono, D., Micheli, M., Facchin, S. L., Tolisano, C., & Proietti, P. (2022). Effects of biogenic ZnO nanoparticles on growth, physiological, biochemical traits and antioxidants on olive tree in vitro. *Horticulturae*, *8*(2), 161.

Rostami, A. A., & Shahsavar, A. (2009). Olive "mission" explants. *Asian J. Plant Sci*, *8*(7), 505-509.

Roussos, P. A., & Pontikis, C. A. (2002). In vitro propagation of olive (Olea europaea L.) cv. Koroneiki. *Plant Growth Regulation*, *37*, 295-304.

Rugini, E., & Fedeli, E. (1990). Olive (Olea europaea L.) as an oilseed crop. In *Legumes and oilseed crops I* (pp. 593-641). Berlin, Heidelberg: Springer Berlin Heidelberg.

Sanchez-Romero, C., 2018. Olive Olea europaea L. In: Jain, S., Gupta, P. (Eds.), Step Wise Protocols For Somatic Embryogenesis of Important Woody Plants. Forestry Sciences. 85, Springer, Cham, pp. 25–38. https://doi.org/10.1007/978-3- 319-79087-9_2.

Sebastiani, L., & Busconi, M. (2017). Recent developments in olive (Olea europaea L.) genetics and genomics: applications in taxonomy, varietal identification, traceability and breeding. *Plant cell reports*, *36*, 1345-1360

Therios, I. N. (2009). *Olives* (Vol. 18). CABI.

Titouh, K., Moussa, K. H., Boufis, N., & Khelifi, L. (2022). Impact of cultural conditions on germination of olive (Olea europaea L.) somatic embryos and plantlets development from the Algerian cultivar Chemlal. *Advances in Horticultural Science*, *36*(3), 185-191.

Varlaro, M. E., Casacchia, T., Alfei, B., & Briccoli Bati, C. (2007, September). In vitro adaptability of some Italian olive genotypes. In *III International Symposium on Acclimatization and Establishment of Micropropagated Plants 812* (pp. 125-128).

WONG, R. S., CHAI, H. X., SUBRAMANIAM, S., & CHEW, B. L. (2021). The establishment of aseptic cultures and multiple shoot induction of Olive (Olea europaea) cv. 1. Malaysian Applied Biology, 50(1), 125-132.

Yancheva, S., Kondakova, V., 2016. Plant tissue culture technology: present and future development. In: Pavlov, A., Bley, T. (Eds.), Bioprocessing of Plant In Vitro Systems, Reference Series in Phytochemistry. Springer, Cham, pp. 1–26. https://doi.org/ 10.1007/978-3-319-32004-5_16-1.

Zohary, D., & Spiegel-Roy, P. (1975). Beginnings of Fruit Growing in the Old World: Olive, grape, date, and fig emerge as important Bronze Age additions to grain agriculture in the Near East. *Science*, *187*(4174), 319-327.