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MICROPROPAGAREA LA SPECIA *PISTACIA LENTISCUS* L. - OPTIMIZAREA PROTOCOLULUI DE STERILIZARE A SUPRAFEȚEI ȘI VENTILAȚIE FORȚATĂ ÎN CULTURA DE IMERSIE TEMPORARĂ

MICROPROPAGATION OF *PISTACIA LENTISCUS* L. - OPTIMIZATION OF THE SURFACE STERILIZATION PROTOCOL AND FORCED VENTILATION IN TEMPORARY IMMERSION CULTURE

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Abstract

***Pistacia lentiscus* L., belonging to *Anacardiaceae* family, is a typical species of the Mediterranean maquis and it is widely grown in Greece and Italy mainly for its aromatic resin extraction or as ornamental plant and also as *Pistacia vera* L. rootstock. Its propagation is difficult either by seed or by cuttings. The current study was carried out to optimize the micro propagation of *Pistacia lentiscus* L. starting from seedlings and woody explants. For the surface sterilization two different protocols were evaluated for woody explant and 6 treatments with combinations of different sterilizing agent types and concentrations were used for mature seeds. For woody explants, no significant differences could be evidenced on contamination percentage and plant survival but the initial growth in vitro of the explant was better in case of the first treatment (1.5% NaOCl for 30 min and 70% Ethanol for 1 min) than opposite combination. The highest seed contamination percentage occurred in case of treatment with 1% NaOCl for 30 min. The treatment with Ethanol (70%) for 30 second followed by three times washing with distilled water then use of NaOCl (1%) for 30 min permitted to obtain 100% of sterility. The highest seed germination (100% after 3 days) was obtained in seeds treated with Ethanol (70%) for 30 second then NaOCl (1%) for 30 min. In order to study the proliferation two different procedures were compared in liquid and agar-based media. Our results proved that proliferation rate increased 6.5 % by forced ventilation system. Longer shoots (10.5 cm) were obtained in temporary immersion system using RITA boxes. This culture system induced also the highest shoot weight which is the increasing of the 29.56% respect common vessels and agar-based medium.**

Cuvinte cheie: arbore de mastic, micropropagare, agenți de sterilizare, cultură de imersie, ventilație forțată.
Key words: lentisk, micropropagation, sterilizing agents, Immersion Culture, forced ventilation

1. Introduction

The lentisk, also known as Mastic tree, (*Pistacia lentiscus* L.), belonging to *Anacardiaceae* family, is grown widely in several Mediterranean countries like Greece and Italy (Zohary, 1952). Lentisk is cultivated mainly for its aromatic resin extraction or as ornamental plant but also as *Pistacia vera* L. rootstock (Acar, 1988; Baytop, 1968). High quality resin is extracted from the lentisk, but it is reported that female plants of lentisk produce low levels of mastic than male ones (Baytop, 1968; Acar, 1988). Essential oil of mastic gum is based on α-pinene (58.86–77.10%), camphene (0.75–1.04%), β-pinene (1.26–2.46%), myrcene (0.23–12.27%), linalool (0.45– 3.71%), and β-caryophyllene (0.70–1.47%). The economic value of mastic resin referred to some important medical and perfumery products. Lentisk secondary metabolites have antioxidant, anti-fungal, anti-bacterial and antimicrobial properties (Calabro and Curro, 1974; Rejeb et al., 2006; Maarouf et al., 2008; Mahmoudi et al., 2010; Mezni et al., 2012). Lentisk like other Pistachio species has long juvenile phase so conventional propagation is difficult. Owing to the high heterozygosity the seedlings are genotypically and phenotypically different from the mother plant Moreover, for the lentisk other asexual propagation methods by grafting, budding, cutting and layering are difficult (Karakir and Sfendiyaroglu, 1999). Micropropagation methods for lentisk are reported by several authors with good results (Ruffoni et al., 2004, Taskın and Inal, 2005). Nevertheless,

serious problems were evidenced for primary sterilization and in vitro multiplication of adult lentisk plants (Mascarello et al., 2007; Yildirim, 2012; Kilinc et al., 2014). It was well documented that, starting from adult plants of Pistachio species like lentisk, leading to a severe occurrence of phenolic compounds and contaminations in vitro at least in the first phase. (Dolcet-Sanjuan and Claveria, 1995; Barghchi and Alderson, 1989). Many attempts were tested for the reduction of the contamination problems for example Onay (2000) proposed meristem tips excision; Parfitt and Almehdi (1994) stated that the reduction of sugar content in the culture medium could prevent contamination. Tilkat et al (2006) showed that the use of 10% NaOCl for 30 min can be effective for surface sterilization of explants on adult Pistachio trees. Different procedures were used to eliminate seeds fungal and bacterial contamination in different plant species (Salehi and Khosh-Khui, 1997; Haldeman et al., 1987; Reed and Tanprasert, 1995; Seckinger, 1995; Sen et al., 2013 a and b). Sen et al (2013) reported that among different sterilizing agents, for seed sterilization of Achyranthes aspera plant, Sodium hypochlorite application for 30 min at 1% concentration was highly effective as a sterilizing agent with 83.44 and 63.88% plant survival, accompanied by 100 and 60% germination percentage. Based on Sen et al (2013) reports, growth and development of explants were not negatively affected by NaOCl treatment, and other procedures of NaOCl treatment involve increased concentration and duration were less efficient also in respect to sterilization, because of negative consequences on growth of plantlets. They also reported that different concentration of sterilizing agents had different effects on contamination level and survival percentage. In a similar study, Tomaszewska-Sowa and Figas (2011) showed that for Cup plant (*Silphium perfoliatum* L.) seeds, among the tested methods, sodium hypochlorite solution proved to be the most effective for disinfestation. Different propagation aspects for lentisk were evaluated by Fascella et al (2004), Ruffoni et al (2004), Mascarello et al (2007) and Taskin and Inal (2005), and mainly have been focused on lentisk explants micro propagation by using agar-based systems. it is well documented that the commercial plant micro propagation in conventional agar-based media is labor intensive and costly (Ascough and Fennel, 2004; Berthouly and Etienne, 2005; Quiala et al., 2012). *In vitro* plant propagation using liquid based media may reduce production costs (Ziv, 2005). Gelling agents increase the production costs and restrict the possibility of automation (Quiala et al., 2012). Furthermore, the liquid culture improves multiplication successes in term of plant quality (Ascough and Fennel, 2004). Despite of all benefits of liquid culture media, the occurrence of hyperhydricity is the main disadvantage of these method (Ascough and Fennel, 2004; Berthouly and Etienne, 2005), So, some techniques were used to prevent this damage for example, membrane rafts, bubble and gassing bioreactors usage, shaking batch cultures applying and temporary immersion system (TIS). Based on previous reports, temporary immersion system has been used for different plant species successfully (Cabasson et al., 1997; Akula et al., 2000; Albarran et al., 2005; Stanly et al., 2010; Yan et al., 2010; Steinmacher et al., 2011 and Yan et al., 2011). Now TIS is used for the production of plant secondary metabolites in some rare herbs. RITA boxes can increase plant growth parameters and propagation yield by ventilation which is in direct relationship with Increasing growth parameters (Ibaraki,1992). In unventilated vessels the plant material shows several physiological disorder, such as inability to photosynthesize, open stomata and lack of a cuticle layer (Fuchigami et al., 1981). It has been demonstrated that in common vessels there is low transpiration rate, minimum water and nutrient uptake, low photosynthesizing enzymes activity and decrease of growth parameters (Jeong et al., 1995). Forced or natural ventilation can create better conditions for plantlets growth by increasing Leaf cuticule layer and also stomatal function (Zobayed et al., 2001). To our knowledge, there are no cited references on Lentisk micro propagation using TIS, furthermore, there is poor information on micro propagation of woody plants using temporary immersion system (Albarran et al., 2005; Yan et al., 2011; Quiala et al., 2012). The present study aims to improve the efficiency of surface disinfestation and initial in-vitro establishment of lentisk by comparing sterilization treatments and in vitro culture initiation of seeds and woody explants from adult lentisk trees. Moreover, In current study in order to develop an efficient micro propagation method for the Lentisk plant, using RITA boxes, a growth comparison versus agar-based media were assessed.

2. Material and methods

2.1. Plant material and sterilization treatments

Woody cuttings were collected in December from lentisk plant, grown at the CREA OF Institute, in Sanremo, a province located in the North of Italy. Immature seeds were collected from plants grown in the wild in Strada Mulattiera San Lorenzo, Liguria region, 163 meter above sea level. Woody explants, 2 cm long with three buds were washed with tap water for 1 hour and were sterilized following the combinations reported in Table 1. Solutions of 1 and 1.5% of sodium hypochlorite (NaOCl) and 70% Ethanol were prepared with distilled water. After sterilization treatment, the basal part of the shoot tips was cut and explants were cultured in the culture initiation medium. The seeds were sterilized according to different treatments (Table 2). The culture initiation medium was Murashige and Skoog (1962) base medium containing mineral nutrients, vitamins, and sucrose (30 g L^{-1}), solidified with Agar (7 g L^{-1}) for woody

explant and (6 g L^{-1}) for seed culture. The pH was adjusted to 5.7 before autoclaving. Woody explants were cultured aseptically in jars containing 40 ml of medium and shelled seeds were cultured in petri dishes containing 25 ml of medium. Data about percentages of contamination survival and growth were recorded after 30 days. After seed sowing Petri dishes were kept in dark condition for 4 days and vessels containing woody explants were kept at 25 °C under a photo period of 16/8 h of cool, white fluorescent light (40 µmol m^{-2} s^{-1}). Data about vitality and contamination of the woody micro-cuttings were recorded after 14 days; visual evaluation of eventual presence of phenolic compounds was also performed and data about germination percentages and days for germination were recorded every 3 days up to 12 days. Final germination percentage (FGP) and mean germination time (MGT) calculated based on Oroiard (1977) method and coefficient of velocity of germination (CVG) calculated according to Jones and Sanders (1987) based on the following equations:

(FGP): final number of seeds germinated/total seed number × 100

(MGT)=$\sum fx/\sum f$, (f: seeds germinated on day x)

(CVG)=$\sum n/\sum nt$, (n: number of seeds germinated each day, t: number of days corresponding to n)

2.2. Proliferation phase using Temporary immersion system (TIS) and agar-based media

Four weeks after the seed germination, shoot tips (seedlings epicotyl) were transferred to WPM media, supplemented with 0.5 mg L^{-1} BA (6-benzyladenine) and 100 mg L^{-1} Ascorbic acid solidified with Agar (7 g L^{-1}) as proliferation medium using non ventilated glass vessels. The Temporary immersion bioreactor containers RITA® vessels (CIRAD VITROPIC, France), containing liquid WPM media supplemented with 0.5 mg L^{-1} BA and 100 mg L^{-1} Ascorbic acid were set at 3hr immersion and 30 min bubbling time and 10 shoot tips of *P. lentiscus* L. were plated in each vessel in forced ventilation. Both treatments (agarised medium and liquid medium in RITA vessels) were maintained in a photoperiod of 16 h of light under cool white light (36 µmol m^{-2} s^{-1}) at 25 ± 2, After 6 weeks, the proliferation rate (number of shoots per initial explant), plantlet height and weight were recorded and analyzed.

2.3. Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means were compared using Duncan's test ($P \leq 0.05$). Before media comparison the percentages were transformed in angular values. For the two different sterilization methods and ventilation treatments, T-tests were used to show differences among treatments. The experimental design was a CRD with 8 replicates, each replicates 1 petri dish containing 3 seeds, and the analysis was performed using SAS 9.

3. Results

3.1. The effects of sterilization method on phenolic compound exudation of woody explants

After 5-7 days from initial establishment, at the base of the Lentiscus woody explants, exudation of phenolic compounds was evidenced together with media in browning and this was the main inhibitor factor for explant establishment. in current experiment exudation of phenolic compounds were not observed as a consequence of different sterilization treatments.

3.2. The effect of sterilization treatments on asepsis of woody explants

The applied treatments improved sterilization percentage of woody explants. The first treatment (immersion in 1.5% NaOCl for 30 min and after treatment with 70% Ethanol for 1 min) showed more contaminations than the second one but this difference was not statistically significant. Furthermore, the treatments had no significant effect on plant survival but the percentage was higher (46.62%) in the first treatment. The explant initial growth expressed in elongation and initial development of the buds was affected by treatments at $P \leq 0.05$, in fact the explants following the treatment 1 grew in the 20.87% respect to the 6.68% after the other one. Contamination percentage remained for both treatments from 30 to 37.5% (Table 3).

3.3. The effect of treatments on seed surface sterilization

According to the results, different sterilization treatments had significant effect on contamination percentage at $P \leq 0.05$, (Table 4). The highest contamination percentage was seen in seeds treated with 1% NaOCl for 30 min (25.56%, treatment 3 - Table 4). Seeds followed treatments 5 and 6 were free from contamination (Table 4) showed also the highest final germination percentage (FGP) in response to the other treatments. It seems that washing before the use of disinfectant agent has negative effect on sterilization procedure. Based on the result, the role Ethanol alcohol 70% in the sterilization is very effective. Our results indicated that usage 1% NaOCl dosage for (30 min) alone, with or without washing treatment cannot have protective effect against contamination agent (Table 4).

3.4. The effects of treatment on FGP, MGT and CVG seed parameters

The treatments had no significant effect on mean germination time (MGT) and on the coefficient of velocity of germination (CVG) but lentisk seeds final germination percentage (FGP) was affected by treatments at P≤ 0.05 (Table 4). As already mentioned the highest seed germination percentage (85.59%) was seen in seeds treated with 70% Ethanol for 30 seconds washed three times by distilled water then 1% NaOCl used for 30 min with no significant difference with revers state (treatments 5 and 6 - Table 4). The minimum value for mean germination time (MGT) was 4.37 days after the treatment 6, followed by 4.56 days after treatment 1. The maximum value for CVG was again after treatment 1 (0.125), which means more speed germination.

3.5. The effect of treatment on development of normal seedlings

Our results demonstrated that different sterilization methods had significant effects on percentage of germination of regular seedlings (with normal root system, stem and leaves) (Table 4). The highest percentage of seedling with a regular behavior was achieved after treatment 6 (45.93% - table 4). The minimum percentage of normal seedlings was achieved after the reverse treatment (treatment 5) indicating that the use of ethanol after NaOCl could inhibit the germination of normal seeds.

3.6. Proliferation treatment results

The different parameters evaluated in the experiment showed a significance at P≤ 0.05. *P. lentiscus* L. shoot tips on liquid WPM media containing 0.5 mg L^{-1} BA and 100 mg L^{-1} Ascorbic acid showed an average of 6.18 shoot explant and plant proliferation rate was increased by 6.5 % using forced ventilation system than agarised medium (Table 5). Agar-based WPM medium containing 0.5 mg L^{-1} BA and 100 mg L^{-1} Ascorbic acid showed an average of 5. 8 shoots explant. Longer shoots (10.5 cm) were obtained in RITA. Plant height in TIS was higher than in agar based medium calculating a significant increase of 16.5%. Temporary immersion system was also more efficient considering the total plant weight (1.49 g, Table 5), mainly due to the high number of shoots per each explant. Plant weight in RITA containers increase by 29.56% in comparison to the other culture types. An example of shoots grown in the two systems is presented in fig. 1.

4. Discussions

According to some published results there have been some problems for primary sterilization and in vitro multiplication of mature lentisk plant materials (Mascarello et al., 2007; Yildirim, 2012; Kilinc et al., 2014) The results we obtained in our work are in agreement with the papers of Dolcet-Sanjuan and Claveria (1995) and Barghchi and Alderson (1989), who reported the extreme difficulty to establish aseptic culture of some *Pistacia* species because of high phenolic compounds level and contamination problems. The browning of the woody explants and the medium surrounding the explant base occurred during initial explant establishment and it is known as the main inhibiting factor for woody explant in vitro establishment especially for the *Pistacia* species (Ozden-Tokatli et al., 2005; Tilkat, 2006). Previous researches proposed different antioxidant treatments for preventing exudation of phenolic compound like PVP, ascorbic acid and other antioxidants; almost no significant differences were observed from the point of browning control view (Wessel et al., 1976; Roy and Sarkar, 1991) but in this study, the preparation procedure of the explants was efficient and permitted to avoid phenolic compound exudation. The results presented in Table (3) showed that either the rate of shoot survival and their initial growth in vitro were high in treatment 5 (immersion in 1.5% NaOCl for 30 min and after treatment with 70% Ethanol alcohol for 1 min) than after treatment 6 which is the reverse treatment that showed also the higher contamination percentage. Our result proved that beneficial effects of NaOCl treatment also on lentisk seed asepsis, and this is in agreement with Sen et al (2013), which reported that between different sterilizing agents, for seed sterilization of another species as *Achyranthes aspera* plant, sodium hypochlorite solution application for 30 min was the most effective treatment and Tomaszewska-Sowa and Figas (2011) which stated that, sodium hypochlorite solution can be the most effective for disinfection of the species *Silphium perfoliatum* L. seeds. Our result showed that seeds treated with 70% Ethanol alcohol for 30 second followed by 1% NaOCl for 30 min were exhibited lower contamination. This emphasize the role of 70% Ethanol alcohol in the disinfection of lentisk seeds. It seems that the use of tap water before sterilizing agents has a negative effect on asepsis procedure which can refer to contamination agent penetration in the seed cuticle. Our results indicated that different sterilizing agents had different effects on the evaluated parameters; for example, final germination percentage (FGP), mean germination time (MGT) and coefficient of velocity of germination (CVG) showed different values. Plant micro propagation using bioreactor system, in the liquid medium is a strategy to prevent some limitations that occurs in plant micro propagation using glass vessels without forced ventilation in agarised substrate. (Paek et al., 2001, 2005). Liquid media could decrease the production costs related to manual labor and enhance the possibility of automation (Quiala et al., 2012). In agreement with our finding, previous result showed that

temporary immersion system performed with RITA containers could improve plant quality and production with high yield in comparison to medium containing agar (Preil 2005). Based on our results, *P. lentiscus* L. shoot tips in liquid WPM medium showed high multiplication rate. The liquid medium that temporary bubble around the shoot tips permitted a high cell hydration inducing longer and heavier shoots rather than agarised medium. In agreement with our finding previous studies showed that bioreactor system used to different plant species successfully, (Cabasson et al., 1997; Akula et al., 2000; Albarran et al., 2005; Stanly et al., 2010; Yan et al., 2010; Steinmacher et al., 2011 and Yan et al., 2011). In agreement with our finding Increasing growth parameters and propagation yield were reported by other researchers by improving ventilation (Ibaraki et al, 1992; Fuchigami et al., 1981; Jeong et al., 1995; Zobayed et al., 2001).

5. Conclusions

To our knowledge, this is the first study on the micro propagation of the lentisk plant by temporary immersion system. Growth parameters comparison between liquid medium and agar-based medium showed that, bioreactor system permitted to increase quality and yield of the plants in proliferation phase. The use of WPM salts confirmed the good performances of the shoots in combination with BA and Ascorbic acid for an effective method for Lentisk micro propagation. The data about the surface sterilization of lentisk seeds and woody explants permitted to establish an efficient procedure for lentisk micro propagation.

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Tables and Figures

Table 1. Sterilization treatments for woody explants of Lentisk (*Pistacia lentiscus* L.)

Treatments number	Phase 1		Phase 2		Rinse (10 min)
	Substance	Exposure time (min)	Substance	Exposure time (min)	
1	NaOCl (1.5%)	30	Ethanol (70%)	1	sterile distilled water (twice)
2	Ethanol (70%)	1	NaOCl (1.5%)	30	sterile distilled water (twice)

Table 2. Sterilization treatments used for Lentisk (P*istacia lentiscus* L.) seeds

Treatments number	Phase 1		Phase 2		Rinse (10 min)
	Substance	Exposure time (min)	Substance	Exposure time (min)	
1	tap water	60	NaOCl (1%)	30	sterile water (twice)
2	tap water	60	NaOCl (1.5%)	30	sterile water(twice)
3	NaOCl (1%)	30	-	-	sterile water (twice)
4	NaOCl (1.5%)	30	-	-	sterile water(twice)
5	NaOCl (1%)	30	ethanol (70%)	1/2	sterile water (twice)
6	ethanol(70%)	1/2	NaOCl (1%)	30	sterile water (twice)

Table 3. The effect of two sterilization methods on contamination, survival and initial growth of *Pistacia lentiscus* L. woody explant evaluated 30 days after treatment

Sterilization treatments	Percent of explants		
	Contamination (%)	Survival (%)	Growth
First NaOCl then Ethanol (treatment 5)	37.5a	46.62a	20.87a
First Ethanol then NaOCl (treatment 6)	30a	40.74a	6.68b
Significance	n.s	n.s	*

*Means followed by the same letters in column are not significantly different (P≥ 0.05).

Table 4. The effects of different sterilization treatments on seed germination (%) and germination speed, contamination percent and normal growth plantlets of *Pistacia lentiscus* L. the value of contamination and percentage of normal plantlets were evaluated after 12 days from treatment

Sterilization treatments	FGP	MGT	CVG	Contamination %	Normal plantlet %
1) Washing (1hr) + 1% NaOCl (30 min)	66.28ab	4.56 a	0.124 a	17.95ab	34.85ab
2) Washing (1hr) + 1.5% NaOCl (30 min)	78.09ab	4.7 a	0.12 a	18.52ab	33.47ab
3) 1% NaOCl (30 min)	61.87b	4.78 a	0.118 a	25.56a	32.89ab
4) 1.5% NaOCl (30 min)	58.02b	5.28 a	0.118 a	16.89ab	28.78ab
5) First 1% NaOCl then 70% Ethanol	85.59a	5 a	0.119 a	0 b	21.91b
6) First 70% Ethanol then NaOCl 1%	85.59a	4.37 a	0.125 a	0 b	45.93a
Significance	*	n.s	n.s	*	*

*Means followed by the same letters in column are not significantly different (P≥ 0.05).
FGP: Final germination percentage; MGT: Mean germination time; CVG: Coefficient of germination speed

Table 5. The effect of forced ventilation in RITA containers versus common glass jars with agar-based medium on proliferation rate, plant height and weight of *Pistacia lentiscus* L

Culture type	Proliferation rate (N. of shoot per explant)	Plant height (cm)	Plant weight (g)
Liquid medium (TIS) with forced ventilation	6.18 a	10.5 a	1.49 a
agar-based medium	5.8 b	9.04 b	1.15 b
Significance	**	**	**

*Means followed by the same letters in column are not significantly different (P≥ 0.05).

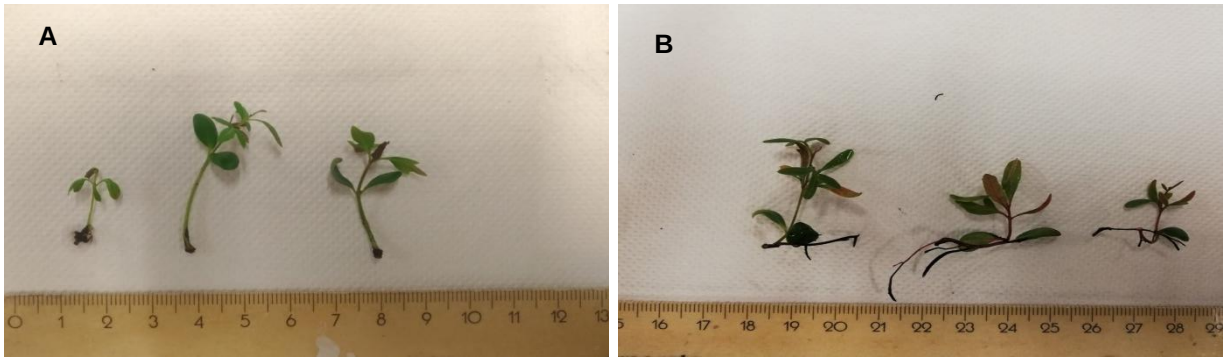

Fig. 1. A - lentisk proliferation phase using RITA containers with forced ventilation; B - lentisk proliferation phase using common jar and agar-based media. 30 days after culture initiation at 25°C and light photoperiod