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Micropropagation of *Pistacia lentiscus* L. from axenic seedling-derived explants

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ABSTRACT

Pistacia lentiscus L. (*Anacardiaceae*), a deciduous forest tree, is important multipurpose *Pistacia* tree. Today, the major limitation facing the widespread expansion of commercial lentisk plantations is the shortage of superior plants primarily because of difficulties experienced in propagating this species by using the traditional vegetative propagation methods. Shoot cultures were established from aseptically germinated seedlings of lentisk. Factors including the different N^6-benzyladenine (BA) concentrations, the combination of cytokinin and other growth regulators, media and antioxidants were assessed and optimized for in vitro shoot proliferation. Full strength MS medium with Gamborg's vitamins containing 30 g l^{-1} sucrose, 100 mg l^{-1} PVP, 1 mg l^{-1} BA and 7 g l^{-1} agar resulted in multiple shoot (bud) initiation at the rate of 2.7 ± 0.17 shoot (4.18 ± 0.17 bud) per explant in 28 days of culture. Moreover, with the use of in vitro proliferated axenic cultures subcultured at least twice, the effects of auxins and mineral medium strength were also assessed for root induction. Efficient rooting (92%) was achieved in a medium containing 1 mg l^{-1} indole-3-butyric acid (IBA). The method developed for plant acclimatization was satisfactory because a high percentage of plant survival (83.33%) in the growth room was obtained and the regenerated plantlets resumed growth after 4 months (96%). The method described will be useful for rapid multiplication of lentisk for commercial exploitation.

1. Introduction

The lentisk or Mastic tree, (*Pistacia lentiscus* L.), a deciduous and dioecious forest tree, is a rustic, evergreen shrub of the Mediterranean maquis. It is native throughout the Mediterranean region from Asian Minor to Canary Islands (Ak and Parlakcı, 2009; Zohary, 1952). In Turkey, the tree is grown especially in Aegean and Mediterranean parts, and it is widespread in forest alone or associated with other tree species such as terebinth, olives and carob etc. in all coastal areas up to 700 m above sea level or in seaside stony areas (Ak and Parlakcı, 2009; Bilgen, 1968). The mastic tree is grown well in several climatic and pedological conditions (Zohary, 1952). It also shows severe tolerance to drought (Correia and Catarino, 1994), and cold (−7 °C) in winter (Anon., 2005) together with resistance to calcareous soil and re-growth after cutting fire injuries (Garcia-Fayos and Molina, 1990; Ladd et al., 2005).

P. lentiscus L. is the unique source of shellac resin obtained from the wounded trunk and thick branches of the male trees due to the low productivity of female ones (Acar, 1988; Baytop, 1968). The aromatic ivory colored resin, also known as mastic (or mastix), is harvested as a spice from the cultivated male trees grown mainly in the South of the Greek island of Chios in the Aegean Sea. Mastic gum from lentisk has been used by traditional healers for the relief of upper abdominal discomfort, stomachaches, dyspepsia and peptic ulcer (Al-Habbal et al., 1984). In the past, *P. lentiscus* L. oil extracted from the mastic gum has been used in several industrial applications such as food, perfumery, lacquers, varnishes and pharmaceutical (Calabro and Curro, 1974), and it has also been re-evaluated as a flavoring in alcoholic beverages and chewing gum (Fernandez et al., 2000). Recent reports have shown that essential oil from aerial parts of *P. lentiscus* L. possesses appreciable biological properties such as antifungal, antibacterial and antimicrobial (Barra et al., 2007; Lamiri et al., 2001).

Although it is not extensive in quantity as on Chios, the tree is native to all of the Mediterranean region, including Çeşme peninsula of İzmir, where the ecological conditions are identical (Acar, 1988; Baytop, 1968). However, mastic tree cultivation has been adversely influenced as a result of diminishing agricultural activities in the recent decades in the peninsula (Baytop, 1968). Currently, *P. lentiscus* L. is naturally propagated by seed with not only consequent increase of genetic variability but also high differences on germination rate among genotypes (Mulas et al., 1998) due to the problems such as parthenocarpy and ovary abortion (Grundwag, 1976). Germination of *P. lentiscus* L. seeds also

Abbreviations: BA, N^6-benzyladenine; DKW, Driver and Kuniyuki; IBA, indole butyric acid; IAA, indole-3-asetic acid; KIN, kinetin; MS, Murashige and Skoog medium; PGR, plant growth regulators; PVP, polyvinyl pyrollidone; QL, Quoirin and Lepoivre; WPM, woody plant medium.

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decreases with the time of storage (Mulas et al., 1998). These characteristics of the seeds reduce the rate of natural propagation. Moreover, cuttings from mature trees of *Pistacia* species are also considered to be very difficult-to-root (Karakır and İsfendiyaroğlu, 1999). They reported that the rooting potential of *P. lentiscus* leafy hardwood cuttings under mist significantly decreased with the cutting collecting times, rooting media and indole butyric acid (IBA) concentrations. Although a high rooting percentage (76.6%) was reported from the cuttings taken in February and treated with 20 g l^{-1} IBA, in perlite (Karakır and İsfendiyaroğlu, 1999), large quantities of mastic tree production was not reported for any reforestation programme. The limiting factor in the propagation of lentisk is the extention of the cultivation. Thus, for its commercial exploitation, there is a need for mass propagation, germplasm preservation, and the production of biologically active compounds. Micropropagation could be an efficient tool in order to avoid difficulties on vegetative propagation and to obtain clonal stocks. Despite the multipurpose use of *P. lentiscus* L., little effort has been made on improvement of lentisk by conventional means or by modern biotechnological approaches. The coverage of literature on this species is mostly restricted to chemical and medicinal aspects (Barra et al., 2007; Ben Douissa et al., 2005; Chryssavgi et al., 2008; Dob et al., 2006; Lo Presti et al., 2008). Plant regeneration through tissue culture techniques has not been reported from juvenile or mature explants of *P. lentiscus* L. However, preliminary studies were reported for the establishment of in vitro cultures of lentisk using in vitro germinated seeds or seedlings (Fascella et al., 2004; Mascarello et al., 2007; Ruffoni et al., 2004; Taşkın and İnal, 2005), but from these studies, in vitro regenerated plantlets or clones were not obtained by in vitro micro-propagation.

It is well-known that the in vitro culture of the mature tree-derived explants is very difficult due to their physiological state. To overcome this obstacle, an alternative approach can be the usage of seedling explants for the standardization of an efficient in vitro propagation in tree species (Giri et al., 2004) which are naturally seed propagated like mastic tree. It is also well known that, in the absence of suitable mature tissues or when older material is non-responsive, the use of mature seeds or isolated zygotic embryos of the control-pollinated plants known to have the desired characteristics may be used for regeneration. Therefore, the aim of this study was to develop an integrated protocol for the micropropagation of seedling-derived apical tips of *P. lentiscus* L. The information reported from this study will help us to develop a reproductive protocol for mass propagation of mature *P. lentiscus* L. plant using in vitro techniques.

2. Materials and methods

2.1. Plant material, culture conditions and establishment of sterile cultures

Mature seeds of *P. lentiscus* L. were collected from natural growing areas in Çeşme County in İzmir province of western Turkey. Mature kernels, from which the outer pericarp had been removed, were surface sterilized by immersion in a 5% (v/v) sodium hypochlorite solution (Commercial Axion) for 5 min. Then, the seeds were washed three times with sterile distilled water before being placed in contact with 50 ml MS (Murashige and Skoog, 1962) medium containing 1.0 mg l^{-1} IBA (indole butyric acid) and 30 g l^{-1} sucrose for germination in Magenta vessels (GA-7, Sigma Ltd.). Four weeks after culture, the mature seeds of lentisk germinated, and the developing seedlings produced actively growing apical shoots. The newly formed shoots were cut into segments containing a node, and these explants were transferred to fresh MS medium supplemented with 1.0 mg l^{-1} BA. By repeating the process of cutting and subculturing twice the shoot tips from in vitro generated shoots, a large number of shoots were proliferated to optimize a novel approach for rapid propagation of *P. lentiscus* L.

All chemicals used were of analytical grade (Sigma Chemical Co., USA). All the explants that were used for the determination of any parameter were subcultured for 4 weeks in a MS (basal MS) medium containing 3% sucrose (w/v) (S5391, Sigma Ltd.) and solidified with agar (0.65%, w/v) (A1296, Sigma Ltd.) in order to eliminate the residuals of growth regulators before using them for the following experiments. The pH of all media was adjusted to 5.8 before autoclaving (120 °C for 20 min). Plant growth regulators (PGRs) were added to the medium prior to adjustment of pH and sterilization. Unless otherwise described, all cultures were maintained in a 16-h photoperiod at 25 ± 2 °C with irradiance of 40 μmol m^{-2} s^{-1}) provided by cool white fluorescent lamps. All experiments were repeated two or three times.

2.2. Effect of media type and strength on shoot proliferation

To study the effect of different media on multiplication and elongation of *P. lentiscus* shoots in culture, shoot tips (approximately 2 cm) were excised and transferred to different full strength media [MS, DKW (Driver and Kuniyuki, 1984), QL (Quoirin and Lepoivre, 1977) and WPM (Lloyd and McCown, 1980)] supplemented with 1 mg l^{-1} BA and 3% sucrose. In a second experiment, in order to examine the effect of medium strength on shoot growth and multiplication, the concentration of nutrients in MS medium was modified from half to full and double strength. Apical shoot tips (subcultured 5 times) were cultured on MS medium supplemented with 0.5 mg l^{-1} BA.

2.3. Effect of BA concentrations on shoot proliferation

Initially, different BA concentrations, 0.5, 1.0, 2.0 and 4.0 mg l^{-1} were used individually in the full strength MS medium to produce shoots and for their further growth and development. Later, on the basis of their responses in culture, the best concentrations were used in combination with previously determined optimum conditions.

The combined effects of 0.5 mg l^{-1} BA plus 0.1 mg l^{-1} IBA and 0.1 mg l^{-1} GA_3 on growth and multiplication of twice-subcultured seedling materials excised from the axenic stock clone of *P. lentiscus* were examined.

2.4. Effect of antioxidants on shoot proliferation

Different antioxidants, like ascorbic acid, citric acid and PVP (each one at 100 mg l^{-1}), were used individually in the full strength MS medium containing 0.5 mg l^{-1} BA to inhibit browning of the basal end of in vitro-proliferating shoots. Later, on the basis of their responses in culture, the best antioxidant was used in cultures.

2.5. Effect of auxin type and medium strength on in vitro rooting

In vitro proliferated shoots (>2.0 cm) were used for rooting experiments. For root initiation, the explants were incubated in Magenta GA-7 vessels, each containing 50 ml of full strength MS medium with auxins including [IAA (indole acetic acid), IBA and NAA (naphthaleneacetic acid)] (Sigma, USA), each at 1.0 mg l^{-1} concentrations was incorporated aseptically in full strength MS medium in GA 7 vessels. MS medium without plant growth regulators served as control. In order to examine the effect of medium strength on rooting, the concentration of nutrients in MS medium varied from quarter to half, full and double strength.