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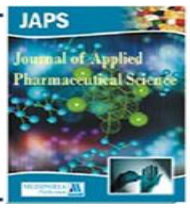

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Micropropagation and shoot regeneration of *Cistus creticus* ssp. *Creticus*

Panagiotis Madesis, Elpiniki Konstantinidou, Athanasios Tsaftaris and Irini Nianiou-Obeidat

Elpiniki Konstantinidou,
Irini Nianiou-Obeidat
Department of Genetics and Plant Breeding, School of Agriculture, Aristotle University of Thessaloniki, P.O. Box 261, Thessaloniki GR-54124, Greece.

Panagiotis Madesis,
Athanasios Tsaftaris
[a]Institute of Agrobiotechnology, CERTH, 6th km Charilaou-Thermis Road, P.O. Box 361, Thermi GR-57001,Thessaloniki, Greece.
[b] *Department of Genetics and Plant Breeding, School of Agriculture, Aristotle University of Thessaloniki, P.O. Box 261, Thessaloniki GR-54124, Greece.*

For Correspondence
Irini Nianiou-Obeidat
Department of Genetics and Plant Breeding, School of Agriculture, Aristotle University of Thessaloniki, P.O. Box 261, Thessaloniki GR-54124, Greece.
Phone +30-2310-998659
Fax: +30-2310-998654

ABSTRACT

Cistus creticus L. ssp. *creticus* is a medicinal aromatic shrub native in Crete (Greece). The protocol described in this paper provides optimal levels of growth regulators required to obtain high regeneration rates of Cistus *in vitro*. Micropropagation has been achieved through rapid proliferation of shoot-tips on Murashige and Skoog (MS) basal medium supplemented with 0.2 mg l^{-1} 6-benzylaminopurine (BAP). After four weeks shoots were transferred to MS medium without growth regulators for further development and rooting. The highest percentage of regenerated shoots was obtained with 0.1 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA after 4 weeks. Elongation and rooting was readily achieved when multiple shoots more than 1 cm in length were singled out and cultured on the MS medium without growth regulators. The plantlets were successfully adapted and grew vigorously in greenhouse conditions. This is the first report of shoot regeneration in the genus *Cistus*. The regeneration protocol developed in this study provides a basis for further investigation of the medicinally active constituents of this elite medicinal plant.

Key words: α-naphthaleneacetic acid (NAA), cretan ladano, shoot regeneration, thidiazuron (TDZ).

INTRODUCTION

Cistus is a genus of flowering plants in the rockrose family *Cistaceae*, containing about 20 species (Ellul et al., 2002). They are perennial shrubs found on dry or rocky soils throughout the Mediterranean region, from Morocco and Portugal to the Middle East, and also on the Canary Islands. The genus *Cistus*, rock rose or holly rose, includes also several medicinal plants (Bouamama et al., 2006). *Cistaceae* species secret essential oils and resins, especially the resin ladano, which is synthesized and stored in modified leaf epidermal glandular trichomes (Demetzos et al., 1997; Pateraki and Kanellis 2008). This compound is used in medicine and perfumery (Demetzos et al., 1999). Leaves of *Cistus salviifolius* and *Cistus incanus* ssp. *creticus* are also used as a substitute for tea (Heywood 1993). All *Cistus* species are commonly used in traditional medicines for their antimicrobial (Demetzos et al., 1999), antitumor (Dimas et al., 2001; Pateraki and Kanellis 2008) and anti-inflammatory properties (Demetzos et al., 2001). Conventional propagation method is the principal means of propagation. Seeds of *Cistus* species have a form of dormancy due to their impermeable seed coat, which is broken when the seeds are exposed to heat. The role of heat shock in the induction of seed germination for numerous Mediterranean fire-following plant species is well documented (Hanley and Fenner 1998). Plant tissue culture is an alternative method of commercial propagation (George and Sherrington 1984), used widely for the propagation of a large number of plant species, including many medicinal and ornamental plants. The plants used in phyto-pharmaceutical preparations are obtained mainly from their natural growing areas. With the increase in the demand for crude drugs, plant populations are being

overexploited, putting the survival of many rare species in jeopardy. Also, many medicinal plants are becoming extinct at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection. This is also a case in *Cistus.* Advanced biotechnological methods of culturing plant cells and tissues could provide new means for conserving and rapidly propagating valuable medicinal plants.

In vitro plant regeneration is a biotechnological tool that potentially could be a solution of this problem, as it provides a means of putting the plants on the market. Moreover, *Cistus creticus* is a recalcitrant species in tissue culture. Only a few studies on the micropropagation of *Cistaceae* have been performed (M'Kada et al., 1991; Morte and Honrubia 1992; Iriondo et al., 1995; Pela et al., 2000), but no method has yet been described to induce shoot regeneration.

In this study, we provide an efficient method for *in vitro* micropropagation from axillary buds and plant regeneration from leaf explants of *Cistus creticus* ssp. *creticus.* Therefore, the regeneration protocol described here is expected to contribute to the future studies in *Cistus creticus*, including molecular breeding and large-scale production of certain biologically active plant metabolites from *in vitro* propagated elite medicinal materials.

MATERIALS AND METHODS

Plant material and *in vitro* shoot culture establishment.

Shoot tips from three-years old ladano plants (clone 26/3) grown at the NAGREF field station, were used in order to establish cultures *in vitro.* Fifty shoot tips were pre-treated with an antioxidant solution (ascorbic acid 100 mg l^{-1}) for 20 min to avoid explants browning (M'Kada et al., 1991). Then the explants were surface sterilized in 100% ethanol for 30 s, rinsed with sterile distilled water and then further sterilized for 15 min in a solution of 1.5% sodium hypochloride (NaClO) with one drop of Triton X-100, and finally rinsed three times with sterile distilled water.

Shoot proliferation

Shoot tips were cultured on MS basal medium (Murashige and Skoog 1962) supplemented with different BAP concentrations (0, 0.2, 0.8, 1.6, 2.0, 4.0, 6.0 mg l^{-1}), 3% (w/v) sucrose and 0.8% (w/v) agar (Agar-Agar, Sigma®) and were sub-cultured every two weeks (Pela et al., 2000). After four weeks, the grown shoots were transferred onto MS medium without growth regulators for shoot elongation and rooting. The *in vitro* produced plants were used as a source of explants for micropropagation and shoot regeneration experiments.

Shoot regeneration from leaf explants

Leaf explants were excised from micropropagated shoots six weeks after *in vitro* culture establishment. The explants used for regeneration were the basal portions with petioles of the second pair of leaves from the shoot tips. Shoot regeneration studies were performed using different combinations and concentrations of growth regulators added in MS medium: 0.1, 0.2, 0.4, 0.8, 1.6, 2.2 and 3 mg l^{-1} TDZ with or without 0.1 mg l^{-1} NAA. Four weeks later, shoot elongation and rooting were achieved when multiple shoots (>1cm) were individually cultured on MS medium without growth regulators. Plantlets were transplanted to 1:1 peat perlit mixture after 3-4 weeks of rooting initiation and acclimatized to the greenhouse environment for three weeks. All cultures were grown at 25 ± 1 ºC under a 16-h photoperiod. To analyze shoot regeneration capability, the regeneration frequency (%) of shoots per regenerating explant was recorded. Four replications, with twelve explants each, were used per experiment. Data were analyzed using a one–way analysis of variance (ANOVA) at 5% probability level and a comparison between the mean values of treatments were made by the least significant difference (LSD) test.

RESULTS AND DISCUSSION

Shoot proliferation

In this study we provide a protocol of an efficient *in vitro* micropropagation method, from axillary buds and shoot regeneration from leaf explants of *Cistus creticus* ssp. *Creticus.*

Micropropagation has been achieved through rapid proliferation of shoot-tips on MS basal medium, supplemented with 0.2 mg l^{-1} BAP. After four weeks, the shoots were transferred onto MS medium without growth regulators for shoot elongation and rooting. The highest frequency of shoot multiplication was 6 shoots per explant, at every 6-week interval.

Few studies on the micropropagation of *Cistaceae* have been done (M'Kada et al., 1991; Morte and Honrubia 1992). Iriondo et al. (1995), developed a micropropagation system applicable to a wide range of genotypes in six rockrose species, initiated from seedlings nodal segments using high concentrations of BAP (5.0 mg l^{-1}) or in combination with NAA and Morte and Honrubia (1992) established a protocol for *in vitro* propagation of *Helianthemum almeriense* Pau (*Cistaceae*). The highest number of shoots per node (7.72) has been obtained on MS medium supplemented with kinetin (an expensive cytokinin) (Morte and Honrubia 1992). Pela et al. (2000) studied the effect of the combination of BAP and indole-3-acetic acid (IAA) or NAA on the proliferation of shoots and on callus induction from two types of explants (shoot tips and lateral buds) taken from *Cistus creticus*. BAP produced 3 shoots per lateral bud or 2.5 shoots per shoot tip after 5 weeks. The combination of BAP and IAA led to callus production after five weeks (Pela et al., 2000). M'Kada et al. (1991) studied the effect of cytokinins on shoot multiplication (6 shoots after 3 weeks) and rooting of *Cistus x purpureus* Lam. Establishment of primary explants *in vitro* was the limiting step because only 55% of the axillary buds were able to develop shoots. Cytokinin levels were shown to be the most critical factor for the proliferation of many medical plants. Similarly, it was observed that cytokinin was required in optimal concentration for shoot proliferation. We were able to establish a simple, rapid and efficient *in vitro* micropropagation protocol from shoot tips of *Cistus creticus*, using MS media supplemented with 0.2 mg l^{-1} BAP (data not shown) which is a very low amount of an inexpensive growth regulator compared to previously reported micropropagation results (M'Kada et al., 1991; Morte and

Honrubia 1992);(Iriondo et al., 1995; Pela et al., 2000). Moreover, the rate of micropropagation using 0.2 mg l^{-1} BAP is higher or similar to those reported for other *Cistus* species. In addition, only Pela et al. (2000) reported micropropagation on *Cistus creticus* but with only 3 shoots per lateral bud in contrast with our protocol were the rate of multiplication is 6 shoots per explant. The effects of auxins and cytokinins on shoot multiplication of various medicinal and ornamental plants were reported by many authors (Martin 2003; Sivanesan et al., 2010; Winkelmann et al., 2010).

Shoot regeneration from leaf explants

Young leaves are known to be a good explant source for shoot regeneration (organogenesis) (Lin et al., 1998; Niu et al., 1998; Phippen and Simon 2000; Phelana et al., 2009). Shoot regeneration was improved when leaves of micropropagated *Cistus creticus* were cultured on MS medium supplemented with different concentrations of TDZ, with or without NAA (Table 1).

Table 1. Effect of growth regulators on shoot organogenesis from leaf explants of *Cistus creticus*.

TDZ (mg l^{-1})	Callus induction (%)	Regenerated shoots (>1cm, %)	Root formation (%)
0.1	95 a	6.9 a	60 a
0.2	92 a	5.4 b	49 b
0.4	92 a	5.8 b	54 b
0.8	83 b	5.0 b	33 c
1.6	79 b	3.0 c	12 d
2.2	75 b	2.4 c	0 e
3.0	72 bc	1.4 c	0 e
TDZ + NAA (mg l^{-1})	Callus induction (%)	Regenerated shoots (>1cm, %)	Root formation (%)
0.1 + 0.1	100 a	33.3 a	100 a
0.2 + 0.1	100 a	27.5 b	100 a
0.4+ 0.1	95 b	6.2 c	98 a
0.8 + 0.1	92 b	5.5 c	95 b
1.6 + 0.1	90 b	5.3 c	95 b
2.2 + 0.1	83 c	4.0 c	92 b
3.0 + 0.1	79 c	2.1 c	90 c

In the columns the figures with the same letters do not differ significantly, four replications, with twelve explants each, were used per experiment. Roots formed either on callus or shoots.

Rapid regeneration through organogenesis from callus culture of cistus was achieved with a wide range of TDZ concentrations when NAA was added (Table 1). Among the treatments, the combination of 0.1 or 0.2 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA produced the highest percentage (100%) of light green in colour and compact callus which has been found to induce shoots after 4 to 6 weeks culture (Figure 1A). The highest frequency of shoot formation (33.3%) was observed when the explants were cultured on 0.1 mg l^{-1} TDZ with 0.1 mg l^{-1} NAA (Figure1B) and were statistically different compared to MS medium supplemented with 0.2 mg.l^{-1}TDZ and 0.1 mg.l^{-1}NAA (27.5%) and all the other treatments. The lowest regeneration frequency was observed with 0.1 mg l^{-1} NAA and 3 mg l^{-1}TDZ, but even these concentrations were not statistically different from concentrations of 0.4, 0.8, 1.6, and 2.2 mg l^{-1}. On the contrary, TDZ alone produced the highest

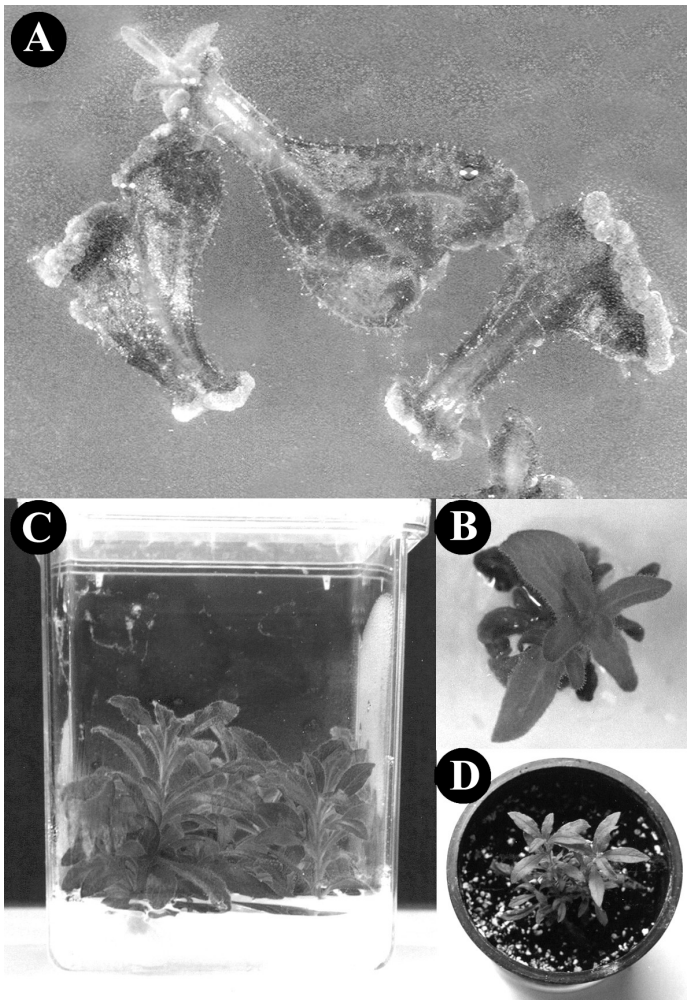

Fig 1 Regeneration from leaf explants of *Cistus creticus* (A): Buds and shoots of *Cistus creticus* regenerated after four weeks on MS medium supplemented with 0.1 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA, bar = 2.5 mm (B): Regenerated and rooted shoots on MS medium without growth regulators after 8 weeks of culture, bar = 10 mm (C): Micropropagation of shoots on MS medium without growth regulators, bar = 24 mm (D): Regenerated plants established in a substrate, bar = 100 mm.

percentage (72-95%) of callus, but only few of these shoot buds have developed to normal shoots (1.4-6.9%). TDZ at 0.1 mg l^{-1} supported low frequency of shoot regeneration (~7 %), but in the other hand was statistically the best concentration of TDZ alone, yet the regeneration frequency was very low and not suitable for a regeneration protocol, whereas higher concentrations (0.2 to 3.0 mg l^{-1}) gave less number of regenerated shoots or were inhibitory for shoot regeneration. Concentrations of 0.2 to 0.8 mg l^{-1} TDZ did not differ significantly amongst them but were significantly different from concentrations of 1.6 to 3.0 mg l^{-1} TDZ (Table 1).

In general, cytokinin induces shoot regeneration (Dai et al., 2009; Pavingerova 2009; Thirukkumaran et al., 2009; Li et al., 2010). Histological evidence has proven that in leaf discs of pigeon pea, TDZ induced *de novo* shoot regeneration (Eapen et al., 1998; Singh et al., 2003). Niu and co-workers have reported shoot organogenesis from peppermint (*Mentha* × *piperita* L.) leaves cultured on MS medium supplemented with BAP, 6γ,γ-dimethylallylaminopurine (2iP) or TDZ (Niu et al., 1998). In addition, regeneration of peppermint was achieved from internodal

segments (Wang et al., 2009). Moreover, studies on organogenesis from various explants supported the perception that MS medium supplemented with TDZ was efficient for shoot induction and plant regeneration in *Sutellaria baicalensis*, *Cajanus cajan* L., *Alstromeria*, *Mentha x piperita* L., *Gypsophilla paniculata Rhododendron simsii*, *Neotchihatchewia isatidea*, *Kalanchoe blossfeldiana*, *Nyctanthes arbour-tristis* L and *Melia azedarach* L (Mertens et al., 1996; Ahroni et al., 1997; Eapen et al., 1998; Lin et al., 1998; Niu et al., 1998; Li et al., 2000; Siddique et al., 2006; Vila et al., 2007).

Shoots longer than 1 cm were individually cultured on MS medium without growth regulators in order to achieve rooting. The excised shoots were elongated and rooted easily on the medium used (Figures 1B and 1C). Rooted shoots were transferred to 1:1 peat:perlite mixture. Three to four weeks later 90 % of plantlets were acclimatized to greenhouse conditions and exhibited normal development. Established plantlets were morphologically similar to the source plant (Figure 1D). This is the first report on *Cistus* regeneration and more specifically on *Cistus creticus* regeneration using leaf explants. The only known reports regarding *Cistus* species are on micropropagation, probably due to the species being recalcitrant in tissue culture.

CONCLUTIONS

We have established an efficient and reproducible method for shoot regeneration of *Cistus creticus*, using leaf explants and TDZ in combination with NAA as a source of growth regulators. The development of a shoot regeneration system for Cretian ladano represents a significant advancement for phytopharmaceutical production of this crop. *In vitro* propagation technology has the potential to provide mass production of sterile, consistent and standardized plant material, which could be utilized for medicinal use through biotechnology methods.

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