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Rapid *in vitro* multiplication of *Cistus ladanifer* L. var. *maculates Dun*.

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Abstract

This work aimed at establishing the protocol for plant regeneration of *Cistus ladaniferus* L.var. *maculatus Dun.* via *in vitro* micro propagation. The nodal explants used came either from cuttings already cultured in media from seed germination or from mature trees. The latter were pre-treated with an antioxidant solution (ascorbic acid 100 mg/L and citric acid 150 mg/L) before sterilization. Explants were cultured on Murashige and Skoog medium (MS) supplemented with 30 g/L sucrose, 8 g/L agar and different concentrations of cytokines (6-Benzylaminopurine (BAP) or(6-(3-methyl-2-butenylamino) purine (2iP)) or of auxins (NAA or IBA) or of their combination. MS medium supplemented with 0,2 mg/L BAP gave the best caulogenic response and the best proliferation of shoots with an average of 1,46 shoots per explants developed on 62,5 % of the cultures after 4 weeks. *In vitro* regeneration shoots developed roots directly from the basal cut ends of shoots without an intervening callus phase, MS medium supplemented with 0.1 mg/L indol butyrique acid (IBA) after 30 days of incubation, 50 % shoots developed roots.In the stage of acclimatization, 70 % of the *vitro*-plants were able to survive in the mixture of garden soil and vermiculite (2:1).In the present study, we established an effective and reproducible protocol for the multiplication of *C. ladanifer*. This study is a basis for the valuing of this medicinal plant.

1. Introduction

The *Cistaceae* family comprises eight genera and about 180 species. They are typically consisting of heliophyte shrubs, subshrubs and herbs occurring in open areas on poor soils [1]. They are pyrophilic, adapted to fires in Mediterranean forests[2].

Cistus L. is a genus of flowering plants in the rockrose family *Cistaceae,* containing about 20 species [3]. 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. Several *Cistus* spp. are used as ornamentals for their flowers, produced in abundance and notable for their size and singular beauty [4].

The genus *Cistus* L.comprises 12 species in the Moroccan flora; including *C. ladanifer*. This species is represented by the only subspecies *C. ladanifer* subsp. *Africanus* Dansereau. In *C. ladanifer*, two varieties are encountered: Var. *AlbiflorusDun*. and Var. *MaculatusDun*[5]. On the branches and flowering tops of this plant, oil-bearing glands produce a kind of gum resin called Labdanum.

This exudateis composed fundamentally of compounds of phenolic and terpene origin [6]. Phenolics and terpenoids are involved in many plant processes, particularly those responding to environmental biotic and abiotic stimuli. Gulz, P. Gproposed that the synthesis of these compounds should be regarded as a defense mechanism of the plant against stress [7].

It is used in folk medicine for treatment of various skin diseases, as an anti-diarrheal and anti-inflammatory agent [8]. In the Moroccan Middle Atlas, *C. ladanifer* is named by the local population as *Touzala*. They are used as an antidiarrheic, antiacid and antispasmodic by simple decoction of their leaves. In addition, anti-diarrheal effect of aqueous extract of *C. ladanifer* has been proved [9]. Furthermore, it produces a number of compounds with pharmacological properties: antihypertensive effect [10], antimicrobial [11; 12], antioxidant [13], antifungal [14].

Cistus spp. multiply naturally by seed, although germination is low due to hard-seededness [15].Vegetative propagation is difficult in most wild *Cistus* species [16]. *In vitro* multiplication of *C. ladanifer* is low and variable

because of a kind of oleaginous gum resin which is synthesized and stored in modified leaf epidermal glandular trichomes,that prevents its proliferation *in vitro* and that progressively causes a browning followed by explants tissue necrosis [17]. So far, a few attempts of the *in vitro* regeneration of *Cistaceae* were achieved. The first attempt on C*istus* nodal segments *in vitro* culture was accomplished by M'Kada et *al*. (1991) [18]. Later on, a protocol of the *in vitro* propagation of *Helianthemum almeriense* (*Cistaceae)* was established by Morte and Honrubia (1992) [19];and a protocol of the *in vitro* propagation of six *Cistus* genuses was improved by Iriondo et *al*. (1995) [4].
Callus induction of *C. creticus* was produced successfully by Zygomala et *al.* (2003) [20]. *In vitro* proliferation of *C. clusii* was also studied with satisfactory results by Ruta and Morone-Fortunato (2010) [21]. A more recent study of *C. creticus* microcomputing distribution was conducted by Madesis et *al*. (2011) [22]. The production of phytohormones and vitamins were studied in *C. albidus* seeds [23], leaves, and flowers [24], in relation to plant maturity. However, few works have been done on the multiplication of *C. ladanifer*.
In this study, we provide an efficient method for *in vitro* micropropagation from axillary buds and plant regeneration fromexplants of *C. ladanifer*var. *maculatusDan*.

2. Material and methods

2.1. Plant material

Our plant samples were collected from the station of Harcha (33°29'1.504" North-latitude, 6°9'2.257" Ouest-longitude,Oulmes, Morocco). The Harcha forest spreads out on a mountainous area. The present study concerned the only variety of this species (Var. *MaculatusDun*.). We used two types of explants in this study: Cuttings already cultured in media from seed germination and cuttings of young shoots from wild plants of the fieldto compare the morphology and yield of the multiplication of each shoot. The latter constitute a wild type and were collected during two years (2012-2014). After ridding shoots of their leaves, they were cut into 1 to 1.5 cm microcuttings of 1 to 2 nodes. These dimensions allow minimizing the risk of getting virus-infected plants.

2.2.Treatment and sterilization of shoots

Sterilization treatments were done under a laminar-flow chamber. The explants from seedgermination and wild plants of the field (nodal segments) were pre-treated with an antioxidant solution (ascorbic acid at 100 mg/L and citric acid at 150 mg/L) for 15 min to avoid explants browning [18]. Then the explants were surface sterilized in 70 % ethanol for 2 min, rinsed with sterile distilled water and then further sterilized for 15 min in a solution of 1.5 % sodium hypochlorite (NaClO) with 3 drops of Tween 80, and finally rinsed three times with sterile deionized (DI) waterduring 15 min each. Distilled water was used for the preparation of the disinfecting solutions and the rinsing. Glass tools and clips were sterilized in the autoclave at 121 °C during 20 min.

2.3. Shoot proliferation

The nodal explants were cultured on MS basal medium (Murashige and Skoog, 1962) [25] supplemented with sucrose at 30 g/L (3% (w/v)) and solidified with agar (Agar-Agar, Sigma®) at 8 g/L. The media were supplemented with different concentrations (0, 0.1, 0.2, 0.4, 0.8 mg/L) of BAP (6-Benzylaminopurine) or (0, 0.1, 0.5, 1, 1.5 mg/L) of 2iP(6-(3-methyl-2-butenylamino) purine).
The media pH was adjusted to 5.8 with 1-N HCl or 0,1-N NaOH, before adding agar. The cultures were done in glass tubes (16 x 180mm) closed with metallic caps and sterilized in an autoclave at 121 °C, under a pressure of 1 bar for 20 minutes. Tubes containing 10 mL of culture media in aseptic conditions were then placed in a culture chamber in which the photoperiod was about 16h of light / 8h of darkness. The temperature was 25 ± 2 °C; and the light, supplied with fluorescent lamps, had an intensity of 1500 Lux (20W/52 blue T12 Philips). After four to five 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.

2.4. Rooting of shoots

Microshoots of about 2-4 cm in length with two or three leaflets, were transferred to MS medium containing various concentrations (0,0.1, 0.5 mg/L) of IBA (Indole-3-butyric acid) or (0, 0.1 ,0.2 mg/L) of NAA (α-naphthaleneacetic acid)or combinations with different concentrations of various cytokines and auxins. 0.5 g/Lactivated charcoal was added to all media.Hormonal combinations used in this experiment areshown in Table 1.

2.5. Acclimatization and transfer of plantlets to soil

After 4 to 5-weeks, rooted shoots were removed from the culture tubes and washed with water to remove media and transferred to pots containing autoclaved vermiculite and soil mixture (1:2). Acclimatization was performed in

the culture chamber under the same conditions as for *in vitro* plant cultures except for a relative humidity near 90% during the first 15 days of acclimatization. Thereafter, these hardened plants were transferred to the greenhouse for 30 days, and then transplanted in the fieldas was done by Gulz, P. G [7], Fennane and Ibn Tattou [5]. The plants were irrigated every other day with deionized water. The culture responses were expressed in terms of explants shooting percentage, number of regenerated shoots or roots per explant, average length of shoots or roots. A completely randomized block design with three replications was used.

Table 1: Hormonal combinations of the various culture media

	Combinations
M_1	MS + 0.1 mg/L (BAP) + 0.2 mg/L (IBA)
M_2	MS + 0.2 mg/L(TDZ) + 0.1 mg/L(NAA)
M_3	MS + 0.1 mg/L(BAP) + 0.5 mg/L(IBA)
M_4	MS + 0.2 mg/L(BAP) + 0.1 mg/L(NAA)

2.6. Statistical analysis

All data were subjected to analysis of variance (ANOVA). Duncan's multiple means comparison test ($P < 0.05$) was performed on the number of shoots per explant. Polynomial regressions were applied to the percentage of rooted microcuttings and the number of roots per microcutting. For each treatment of the transplanting experiment, 24 explants were cultured.

3. Results and discussion

In this study we provide a protocol of an efficient *in vitro* micropropagation method. The initial cultures of the explants derived from the mature shrubs. After successful decontamination of cultures, the other major constraints were the slow growth and low percentage of morphogenic response because of a kind of oleaginous gum resin that prevents *in vitro*proliferation of shootsand that progressively causes a browning followed by explants tissue necrosis (Fig. 1A). The pretreatment with an antioxidant solution (ascorbic acid 100 mg/L and citric solution 150 mg/L) for 15 min avoided explants browning (Fig. 1B).

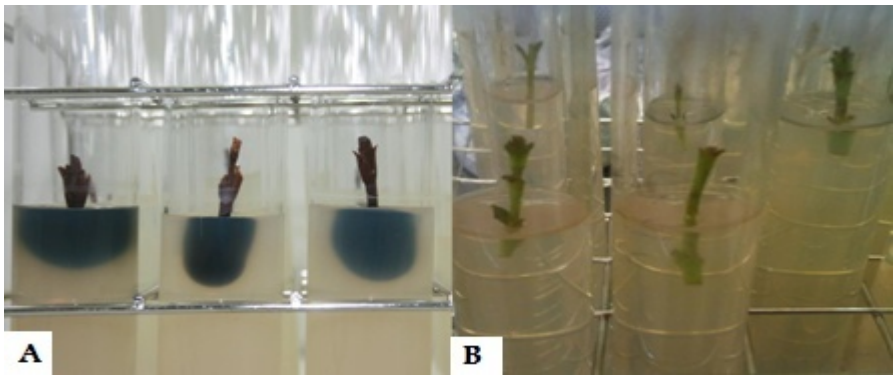

Figure 1: Multiplication of *C. ladanifer*(**A**: without treatment, **B**: Treatment by an antioxidant solution)On MS basal medium, the axillary buds from wild plants of the field proliferated to shoots after 4 weeks of culture in 13.6 % of the cultures (vs. 20,8% for seed germination).

The supplementation of MS medium with different concentrations (0.1, 0.2, 0.4, 0.8 mg/L) of BAP and (0,0.1, 0.5 mg/L) of IBA enhanced the morphogenic potential of explantsfrom wild plants of the field.At 0.2 mg/L BAP and 0.1 mg/L IBA, 50 % of the cultures developed an average 1.27 shoots per explant (Fig2A). Addition of BAP and IBA to the medium not only enhanced the multiplication rate but also favored the proliferation of healthier shoots. Owing to the low caulogenic response from these nodal explants, the further experiments were carried out using microcuttings derived from the shoots regenerated only from the micropropagated cultures.

In order to maximize the shoots proliferation capacity of the nodal explants derived from the microshoots, different concentrations (0.1, 0.5, 1, 1.5 mg/L) of 2iP and (0.1, 0.2, 0.4, 0.8 mg/L) of BAP were applied. All the used concentrations of BAP produced multiple shoots of the explants. However, 0.2 mg/L proved to be optimal in more than 62.5% of the explants, producing an average of 1.46 shoots per explants with an average shoot length of 1.96 cm (Table 2, Fig.2B). The same observation was reported by Madesis *et al.* (2011) for *Cistus creticus* ssp. *Creticus* at low BAP concentration.Effect of different concentrations of 2iP on shooting of microcutting of *Cistus ladanifer* L (Table 3), shows that 2iP concentrations did not affect shoot induction ($p>0.05$).

Table 2: Effect of different concentrations of BAP (mg/L) on shooting of microcutting of *Cistus ladanifer* L.

BAP(mg/L)	Percentage of response (%)	Number of shoots /micro cutting (Mean ± SE)	Length of shoot (cm) (Mean ± SE)
0,2	62,5^{a}±0,084	1,458^{a}±0.282	1,958^{a}±0.375
0,8	41,7^{ab}±0,103	1,083^{a}±0.294	1,246^{ab}±0.346
0,4	41,7^{ab}±0,103	1,083^{ab}±0.294	1,075^{ab}±0.277
0,1	37,5^{ab}±0,101	1,000^{ab}±0.318	1,058^{ab}±0.309
0	20,8^{b}±0,084	0,542^{ab}±0.240	0,554^{b}±0.230
Pr > F	0,066	0,275	0,038

Mean values,within a column, lacking a common letter are significantly different (Duncan, P≤0.05).

Table 3: Effect of different concentrations of 2iP (mg/L) on shooting of microcutting of *Cistus ladanifer* L.

2iP(mg/L)	Percentage of response (%)	Number of shoots /micro cutting Mean ± SE	Length of shoot (cm) Mean ± SE
0,1	29,2^{a}±0,094	0,583^{a}±0,207	0,717^{a}±0,244
0,5	25,0^{a}±0,090	0,458^{a}±0,180	0,554^{a}±0,214
0	20,8^{a}±0,084	0,542^{a}±0,240	0,554^{a}±0,230
1	20,8^{a}±0,084	0,458^{a}±0,200	0,475^{a}±0,200
1,5	20,8^{a}±0,084	0,333^{a}±0,143	0,483^{a}±0,206
Pr > F	0,948	0,917	0,941

Mean values,within a column, lacking a common letter are significantly different (Duncan, P≤0.05).

At higher levels of BAP, though there was no significant difference (p≤0.05) in percentage of explants developing shoots, the average number of shoots per explants decreased significantly (p≤0.05).The effect of auxins, in particular IBA and NAA, on the rate of rooting and on the roots, length depended not only on the nature of the growth regulator but also on its concentration in the medium.Three-to-four-cm-long regenerated shoots were transferred to rooting mediumon the MS basal medium.The induction of roots took place in 16.7 % of the cultures after 4 weeks. To enhance the rooting response, MS medium was further supplemented with concentrations (0,0.1,0.2 mg/L) of NAA (Table 4). On IBA-supplemented medium at (0, 0.1,0.5 mg/L), root development was accompanied with callus formation.However, the 0.1 mg/L IBA-supplemented medium exhibited the best response with a fragile root system(Table 5, Fig.2C). At this concentration, 50 % of shoots developed roots with an average of 1.5 roots per shoot. At higher concentrations of IBA, both percentage of root-forming shoots and number of roots decreased significantly (p≤0.05).

Table 4: Effect of different concentrations of NAA (mg/L) on rooting of microcutting of *Cistus ladanifer* L.

NAA (mg/L)	Percentage of response (%)	Number of roots/micro cutting Mean ± SE	Length of root (cm) Mean ± SE
0	16,7^{a}±0,077	0,292^{a} ±0,153	0,267^{a}±0,137
0,1	16,7^{a}±0,077	0,250^{a}±0,124	0,250^{a}±0,120
0,2	0,83^{a}±0,057	0,125^{a}±0,091	0,083^{a}±0,059
Pr > F	0,639	0,622	0,438

Mean values followed by the same letter in a column are not significantly different (Duncan, P≤0.05).

Table 5: Effect of different concentrations of IBA (mg/L) on rooting of microcutting of *Cistus ladanifer* L.

IBA (mg/L)	Percentage of response (%)	Number of roots/micro cutting Mean ± SE	Length of root (cm) Mean ± SE
0,1	50,0^{a}±0,104	1,500^{a}±0,340	1,392^{a}±0,335
0,5	29,2^{ab}±0,094	0,583^{b}±0,207	0,554^{b}±0,205
0	16,7^{b}±0,077	0,292^{b}±0,153	0,267^{b}±0,137
Pr > F	0,043	0,003	0,004

Mean values followed by the same letter in a column are not significantly different (Duncan, P≤0.05).

The combination of concentrations of various cytokines, in particular BAP and TDZ with various concentrations of auxins, in particular IBA and NAA, gave different results.
However, we found that medium M_1(MS + 0.1 mg/L (BAP) + 0.2 mg/L (IBA)), was the highest (though not significantly) in percentage of response (37.5%), number of roots per micro cutting (0.833) and number of shoots per micro cutting (0.875) (Table 6, Fig.2D). The effect of hormones, in particular IBA and BAP, on the percentage of response, number of roots per micro cutting and number of shoots per micro cutting depended not only on the nature of the growth regulator but also on its concentration in the medium.

Table 6: Effect of different combinations with different concentration of hormones (mg/L) on rooting and shooting of microcutting of *Cistus ladanifer* L.

Combinations with different concentration of hormones	Percentage of response (%)	Number of roots/micro cutting Mean ± SE	Number of shoots /micro cutting Mean ± SE
M_1	$37,5^a$±0,100	$0,833^a$±0.253	$0,875^a$±0.331
M_2	$33,3^a$±0,099	$0,833^a$±0.253	$0,750^a$±0.284
M_3	$33,3^a$±0,099	$0,542^a$±0.180	$0,625^a$±0.268
M_4	$25,0^a$±0,090	$0,458^a$±0.180	$0,375^a$±0.157
Pr > F	0,831	0,502	0,594

Mean values followed by the same letter in a column are not significantly different (Duncan, P≤0.05).

Our results comply with those of Iriondo et *al*, M'kada et *al* and Morte et *al*[4;18;19] about the *in vitro* multiplication of *Cistaceae*. Concerning auxins,we noticed that their importance was in obtaining a high percentage of rhizogenesiswith the addition of IBA at 0.1 mg/L. Indeed, we found a higher multiplication rate with the addition of BAP at 0.2 mg/L.The combination of 0.1 mg/L of BAP with 0.5 mg/L of IBA, gave the best results. Madesis et *al.* (2011) [22] showed that the highest percentages of regenerated shoots were obtained with 0.1 mg/L of TDZ and 0.1 mg/L of NAA. Pela et *al,*[26] studied the effect of the combination of BAP and IAA or NAA on the proliferation of buds and on the induction of calluses from two types of explants (the shoot tips and side buds) taken from *C. creticus*. ,the lowest concentrations give a significant result, confirm our results.Concerning acclimatization, the transferred plantlets were initially kept in the culture chamber for 1-2 weeks, until they started developing new leaves. Of the transferred plants 80 % plantlets survived in the culture chamber (Fig. 2E). After 2 weeks of incubation in the culture chamber, the pots were transferred to the greenhouse where more than 70 % of the plantlets survived (Fig. 2F).

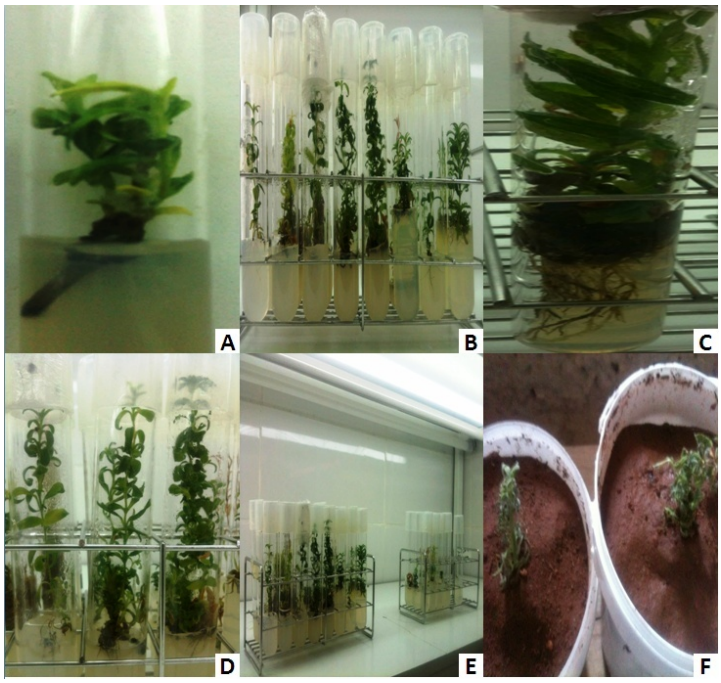

Figure 2 : *In vitro* multiplication of *C. ladanifer* **A**: cuttings of young shoots from wild plants of the field at MS - **B**: MS + 0.2 mg/L of BAP produced multiple shoots of the explants - **C**: MS + 0.1 mg/L of IBA- **D**: M_1 **(**MS + 0.1 mg/L (BAP) + 0.2 mg/L (IBA)), - **E**:culture chamber -**F**: Acclimatization.

Micropropagation would ensure a continuous supply of plants in limited time and space, thereby ruling out the dependence on natural substrates to fulfill the growing demands for the plant material.

Conclusion

We have established an efficient and reproducible method for shoot multiplication of *C. ladanifer*, using two types of explantsfrom seed germination and from wild plants of the field. However, owing to the low caulogenic response from explants of the field, experiments were carried out using microcuttings derived from the shoots regenerated from the micropropagated cultures.The development of a shoot regeneration system for *C. ladanifer* represents a significant advancement for industrial 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 purposes, through biotechnology methods.

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