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New Insights on *Cistus salviifolius L.* Micropropagation

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Research Article

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ABSTRACT

One of the major concerns in the establishment of any mycorrhization program is ensuring the mass production of sterile, consistent and standardized plant material. In the present study, a successful protocol for micropropagation of *Cistus salviifolius L*. was developed. The process was initiated from nodal segments excised from mature *C. salviifolius* plant selected due to its mycorrhizal capacities. Murashige and Skoog basal medium supplement with gibberellic acid (0.5 mg/L) and of 6-Benzylaminopurine (0.5 mg/L) was the best medium for proliferation purposes and successful rooting was achieved with the same basal medium supplemented with Indole-3-butyric acid (0.5 mg/L). The proposed methodology represent a novelty because it allowed the rapid multiplication of *C. salviifolius* starting from mature explants, here reported for the first time, using lower plant growth regulators concentrations than the previously reported for this particular *Cistus* species

INTRODUCTION

The genus *Cistus* L. (*Cistaceae*) is one of the most characteristic genera of the Mediterranean flora [1]. It encompasses a group of about 20 perennial shrub species, distributed throughout the Mediterranean region and Canary Islands, all sharing the same distinctive feature, a combination of diverse hair types on the leaf, stem, and calyx [2,3]. *Cistus* species exhibit a range of specific adaptations as well to Mediterranean environments, such as, fire-dependent seed germination, insect-dependent pollination, flower-dependent reproduction and spring-dependent phenology [4].

Cistus species are involved in many ecological processes taking place in Mediterranean ecosystems [5]. Furthermore, they support a vast and rich mycobiota, constituting reservoirs for mycorrhizal fungal inoculum in the absence of host trees [6]. In total, more than 200 fungal species, belonging to 40 genera, have been reported to be associated with *Cistus*. Among which, several edible hypogeous *Ascomycota*, mainly included in *Tuber* and *Terfezia genera*, and commonly known as truffles [7]. Truffles are highly sought-after and some species command extraordinary prices in local markets, however, due to their ectomycorrhizal nature, truffles must be cultivated in orchards with their plant hosts [8]. As proposed by Giovannetti and Fontana, the wide variety of *Cistus* (and other *Cistaceae*) environmental and ecological requirements makes them ideal candidates to increase the range of habitats where truffles can be grown [9]. Thereby, inoculating these *Cistaceae* with truffle inoculum and planting them in a primary stage of truffle forest repopulation, has become an extremely interesting new use for *Cistus* plants and one with great economic importance and potential for forestry purposes [10].

Cistus salviifolius L. a low subshrub up to 1 m tall, with ovate to rounded leaves and white flowers is the most widely spread species of the *genus* around the Mediterranean basin [3]. It can occur in sandy soils over a wide range of habitats and has been regularly reported as a plant host for various *Terfezia* species, which makes it one of the best choices for planned *Terfezia* cultivation over a wide range of habitats [7,11,12].

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Conventional propagation methods are still the main means for obtaining many ornamental *Cistus* varieties [13]. Nevertheless, vegetative propagation proved to be problematic when wild varieties were used, so *in vitro* micropropagation approaches begun to be tested in the nineties, to overcome the problem of clone production from selected individuals [14-18]. It is widely accepted that tissue culture techniques can represent a reliable and feasible alternative for the rapid multiplication and production of true-to-type plants in limited space and time [19]. The success and efficiency of these *in vitro* micropropagation techniques is influenced by many factors, such as, plant genotype, the physiological status of the explants, culture medium and plant growth regulators (PGRs) [20].

In what concerns *Cistus* micropropagation, one of the most important factors, especially in the shoot proliferation stage, is the amount of cytokinin hormone. Indeed, M'Kada et al. working with nodal segments, excised from mature plants of *Cistus* × *purpureus* Lam., observed that the *in vitro* establishment of the initial explants represented a limiting step, since half of them were unable to develop new shoots. Cytokinins are known to delay senescence, promote mitosis, and stimulate differentiation of the meristem into shoots and roots [20]. Thus, in early works, high concentrations of cytokinins were experimented in order to stimulate the proliferation of new shoots excised from seedlings with satisfactory results [3,13]. Despite these early experiments resulted in successful micropropagation of various *Cistus* species, among which of *C. salviifolius*, it is known that at high levels cytokinins tend to induce callusing, which can utterly compromise the clonal nature of the micropropagated plants and inhibit the elongation of individual shoots [15,20]. In such cases, the addition of gibberellic acid (GA3) to the plant tissue culture media, has been shown to diminish or prevent the formation of somatic embryos, adventitious roots or shoots and promote internode extension and enhance apical dominance [21]. Recently, improved protocols for shoot regeneration using shoot tips of mature *Cistus* plants using only small amount of PGRs have been developed for *C. creticus* and *C. clusii* [13,22].

To our best knowledge, no report was published to date on the shoot regeneration of *C. salviifolius* with high ability to mycorrhize with *Terfezia*, using low concentrations of PGRs and starting from mature explants. Therefore, the aim of the present work was to establish a rapid and optimized *in vitro* micropropagation protocol for rapid multiplication and production of true-to-type *Cistus salviifolius* plants, thus allowing its application for mass production of mycorrhized plants and ultimately enabling *Terfezia* cultivation over a wider range of habitats.

MATERIALS AND METHODS

Plant Material

C. salvifolius plantlets growing in Herdade da Mitra, near Évora (Alentejo, Portugal) (38°32'N; 8°01'W; 220 m a.s.l.)., were collected on November 2013 in a Montado area with natural shrub undercover dominated by *Cistus* spp. The area belongs to the Mediterranean pluviseasonal-oceanic bioclimate and is located in the low mesomediterranean bioclimatic belt. It has a dry to subhumid ombrotype with a mean annual temperature ranging from 9.2°C to 21.5°C and a mean annual rainfall of 664.6 mm [23,24]. All *C. salvifolius* plantlets were washed and disinfected twice with a bleach solution (1% NaOCl (w/v)), potted in sterile substrate (sand, vermiculite, soil; 1:1:1) and placed in a grow chamber for 30 days (24°C/21°C (+1°C) day/night temperature and 15 h light period, under cool white fluorescent light (36 µmol-$m^{-2}s^{-1}$). The plantlets were inoculated with *Terfezia arenaria* spores obtained from dry sporocarps stored in the UEVH Fungi Herbarium. Plant survival and mycorrhization rates were evaluated three months after inoculation, according to the protocol proposed by Giovannetti and Mosse [25]. Ninety three percent of the plantlets survived, and of those 82% were successfully mycorrhized with *T. arenaria*. All plantlets were maintained under those artificial growth conditions, for 24 months, until become adult plants. After that period, mycorrhizae persistence was evaluated and the plant that showed higher micorrhization rate (95%) was chosen to be the source of the initial explants for the *in vitro* culture. Single node segments, each with two opposite buds, were excised from actively growing shoots.

Explant Sterilization

Explants- single nodal segments- were surface sterilized in a four-step procedure: 1) immersion in ethanol (70%) for 2 min; 2) one rinse in bi-distilled water; 3) immersion in $CaCl_2O_2$ (1%) with eight drops of Tween 20 for 20 min; 4) three rinses with bi-distilled water.

Shoot Proliferation

During the culture establishment phase, the authors observed that *C. salviifolius* explants grown in MS basal medium without growth regulators did not produced new shoots. Furthermore, the explants shown hyperhydricity symptoms and stunted appearance, leading to high mortality rates and low multiplication rates. The later problems were solved with the addition of 0.5 mg/L of gibberellic acid (GA3) to the basal media (data not shown). However, shoot proliferation rate continued to be unsatisfactory to our purposes [26]. Thus, for proliferation purposes, it was necessary to test different media formulation and to ascertain if the addition of a cytokinin would improve the production of new shoots. Bearing that in mind, two basal media: MS and WPM, both supplemented with GA3 (0.5 mg/L) with or without 6-Benzylaminopurine (BAP) (0.5 mg/L) were tested, namely: MSG (MS+0.5 mg/L GA3), MSGB (MS+0.5 mg/L GA3+0.5 mg/L BAP), WPMG (WPM+0.5 mg/L GA3), WPMGB (WPM+0.5 mg/L GA3+0.5 mg/L BAP) [27].

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The experiment was conducted with 50 explants per treatment, 10 explants in each of the five culture flasks, in a total of 200 explants. The explants were subcultured every 30 days to fresh medium during three months. Cultures were kept in a growth chamber with 24°C/21°C (+1°C) day/night temperature and 15 h light period, under cool white fluorescent light (36 $\mu molm^{-2}s^{-1}$). At the end of the experiment, the number of new shoots and the number of nodes per shoot were determined. Proliferation rate was evaluated considering the number of shoots per explant.

Rooting

Given that the explants did not form roots in the previous media formulations, a trial for rooting purposes was conducted. MS basal medium was chosen since it proved to be the best medium in the shoot proliferation stage. To induce plant rooting two approaches were taken in consideration: 1) the direct addition of auxins to the media and 2) the promotion of the natural production of auxins by the explants. For that purpose, different formulations were tested using MS basal medium, supplemented with or without activated charcoal and/or Indole-3-butyric acid (IBA), namely: MSC (MS+0.2% of activated charcoal), MS0.1 (MS+0.1 mg/L IBA), MS0.5 (MS+0.5 mg/L IBA), MS0.1C (MS+0.1 mg/L IBA+0.2% of activated charcoal), MS0.5C (MS+0.5 mg/L IBA+0.2% of activated charcoal).

The trial was conducted using 50 explants per treatment, 10 explants in each of the five culture flasks, in a total of 250 explants. The explants were subcultured every 30 days to fresh medium during three months. During that time, cultures were kept in a growth chamber with 24°C /21°C (+1°C) day/night temperature and 15 h light period, under cool white fluorescent light (36 $\mu molm^{-2}s^{-1}$). At the end of the experiment, the number of roots and the tap root length of each explant were recorded. The rooting rate was evaluated considering the number of roots per explant.

Statistical Analysis

The experiments were conducted under a complete randomized block design and data behavior was evaluated by ANOVA analysis. Differences within and between treatments were estimated by mean of separation analysis, using the least significant difference also prevented hyperhydricity in the new shoots and/or leaves (data not shown), allowing the successful establishment of *C. salviifolius in vitro* culture.

RESULTS

Shoot Proliferation

C. salviifolius explants, cultured on media without growth regulators or only with cytokinin, did not thrived, showing hyperhydricity symptoms and stunted appearance. The addition of 0.5 mg/L GA3 to the basal media, not only improved shoot elongation but also prevented hyperhydricity in the new shoots and/or leaves (data not shown), allowing the successful establishment of *C. salviifolius in vitro* culture.

Culture establishment was overall more efficient with MS formulations than with WPM, with significant differences concerning both, the number of nodes per shoot and number of shoots per explant. Furthermore, the highest shoot proliferation rate was achieved in MS supplemented with 0.5 mg/L BAP **(Table 1)**, and thus the best media for multiplication purposes was MSGB.

Table 1. Proliferation rate (shoot number per explant), shoot length and the number of nodes per shoot on the four-tested media (MSG: MS+0.5 mg/L GA3; MSGB: MS+0.5 mg/L GA3+0.5 mg/L BAP; WPMG: WPM+0.5 mg/L GA3; WPMGB: WPM+0.5 mg/L GA3+0.5 mg/L BAP). Means followed by the same letters are not significantly different at $p \leq 0.05$.

Variables	MSG	MSGB	WPMG	WPMGB
n°shoots/explant	2.96+0.22[a]	4.32+0.19[b]	2.06+0.17[a]	2.90+0.19[a]
Shoots length (cm)	3.07 ± 0.15[a]	3.27+0.13[a]	2.98+0.12[a]	3.09+0.12[a]
n°nodes/shoot	5.32+0.63[a]	5.90+0.87[a]	4.35+0.51[b]	4.70+0.48[b]

Rooting

Root induction were successfully achieved in all tested medium. The highest rooting rate (8 roots/explant) was observed in MS basal medium supplemented with 0.5 mg/L IBA **(Table 2)**, with significantly production of more and longer roots. The addition of activated charcoal alone induced the formation of roots in more than 70% of the explants, but in a few number and length, not assuring the plant survival in the next steps. Moreover, the simultaneously addition of charcoal and IBA did not improved the rooting rates.

Table 2. Rooting rate (n° roots/explant) and tap root length on the five-tested media (MSC: MS+0.2% of activated charcoal; MS0.1: MS+0.1 mg/L IBA; MS0.5: MS+0.5 mg/L IBA; MS0.1C: MS+0.1 mg/L IBA+0.2% of activated charcoal; MS0.5C: MS+0.5 mg/L IBA+0.2% of activated charcoal). Means followed by the same letters are not significantly different at $p \leq 0.05$.

Variables	MSC	MS0.1	MS0.5	MS0.1C	MS0.5C
n°roots/explant	1.43+0.10[a]	2.24+0.23[a]	8.04+0.65[b]	2.01+0.19[a]	2.09+0.18[a]
Tap root length (cm)	0.83+0.10[a]	1.16+0.08[a]	1.71+0.11[b]	1.07+0.14[a]	1.18+0.14[a]
Rooted explants (%)	77	83	85	67	73

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DISCUSSION

One of the major concerns in the establishment of any mycorrhization program is ensuring the mass production of sterile, consistent and standardized plant material. Tissue culture techniques have the potential to overcome the problem of clonal production from selected individuals, as they provide the means to rapidly multiply and produce true-to-type plants. Reports on the *in vitro* propagation of *Cistaceae* are still scarce and so far, only Iriondo et al. described a micropropagation system applicable to *C. salviifolius* starting from nodal segments excised from seedlings, using high concentrations of BAP [13]. In this study we tackled these issues and developed the first *in vitro* micropropagation protocol for shoot regeneration and rooting from mature *C. salviifolius* plants. Furthermore, our study show that is possible to obtain similar proliferation rates as those observed by Iriondo et al. [15], using smaller amounts (0.5 mg/L) of 6-Benzylaminopurine (BAP) thus reducing the risk of somaclonal variation. One noteworthy difference was the need to add 0.5 mg/L GA3 in the culture establishment and shoot proliferation stages, to improve shoot elongation and prevent hyperhydricity. One possible explanation for this fact is the genetic traits of the selected plant, whose metabolic pathways might be slightly different from the others. The selected plant showed remarkable mycorrhizal abilities and it is known that ectomycorrhizal fungi can produce and release phytohormones, among which GAs [28-30]. Therefore, it is possible that the addition of GA3 to the media aided to simulate the natural conditions, which might have favored the plant establishment.

The rooting rate obtained with MS0.5 represents an improvement compared with the previous work of Iriondo et al. [15], which obtained a rooting rate of 4.4 roots/explant using IBA (≈1.0 mg/L). The addition of charcoal did not improve the root production, in fact charcoal diminish the rooting rate probably due to the inhibition of plant IBA uptake.

CONCLUSION

In summary, the present work proposes a new methodology which allow the rapid multiplication of *C. salviifolius* starting from mature explants, using lower plant growth regulators concentrations than the previously reported for this particular *Cistus* species. This *in vitro* micropropagation protocol can be useful for multiplication and production of selected *Cistus salviifolius* genotypes, particularly when the purpose is the mass production of plant material for mycorrhization assays.

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