Plant Cell Tiss Organ Cult (2011) 107:531–540
DOI 10.1007/s11240-011-0004-x

ORIGINAL PAPER

GIS-facilitated in vitro propagation and ex situ conservation of *Achillea occulta*

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Received: 24 February 2011 / Accepted: 4 July 2011 / Published online: 26 July 2011

Abstract A Geographical Information Systems (GIS)-facilitated approach for the in vitro propagation and ex situ conservation of the conservation priority species *Achillea occulta* is presented. To realize the species' ecological requirements, the coordinates of the original habitat were linked with thematic layers derived from digital databases in a GIS environment. From wild plants, shoot tips were established in vitro in a basal medium with 4 μM 6-benzyladenine (BA) and 0.5 μM indole-3-butyric acid (IBA). A modified basal MS medium (modMS, double amount of Fe) proved to be the most effective for in vitro adventitious shoot production. The effect of BA in combination with α-naphthaleneacetic acid (NAA) or IBA on shoot proliferation was also tested. The highest number of new microshoots/explant (3.5), with 0.93 cm shoot height was obtained when the modMS was supplemented with 5 μM BA and 2.5 μM IBA. To evaluate the root induction ability, microshoots produced were transferred to modMS media supplemented with 0–20 μM IBA and 0–20 μM NAA. Rooting proved to be very difficult and only by adding 20 μM IBA, a 12.5% rooting percentage was achieved. Acclimatization succeeded only during early spring. Young plants transplanted at the Balkan Botanic Garden of Kroussia produced flowers and seeds in the first year. This GIS-approach provided useful guidelines for *A. occulta*'s (a) effective propagation (selection of greenhouse temperatures, temperatures during in vitro culture, suitable period for cuttings and acclimatization of plantlets), and (b) ex situ cultivation (selection of watering regime, temperatures, locations and exposures for growing sites).

Keywords Auxin · Biodiversity · Cytokinin · Endemic · Protocols · Threatened

Abbreviations

B5	Gamborg medium (Gamborg et al. 1968)
BA	6-benzyladenine
BBGK	Balkan Botanic Garden of Kroussia
GIS	Geographical Information Systems
GSPC	Global Strategy for Plant Conservation
ESPC	European Strategy for Plant Conservation
IBA	Indole-3-butyric acid
IPS	Important Plant Species
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthaleneacetic acid
WPM	Woody Plant Medium (Lloyd and McCown 1980)

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Introduction

In the framework of the Convention on Biological Diversity (CBD 1992) and the global efforts to halt the biodiversity loss by 2010, the Balkan Botanic Garden of Kroussia (BBGK) has focused exclusively to the native Greek flora within the Balkan and European context. Located at a biodiversity 'hot-spot' (the Greek peninsula in the Mediterranean basin) and aiming to contribute to the

implementation of the Global and the European Strategies for Plant Conservation (ESPC 2009; GSPC 2002), the BBGK has formulated a clear conservation strategy (Maloupa et al. 2008; Maloupa and Krigas 2008). This strategy is primarily targeted to the unique floristic elements known to occur in Greece i.e. local, regional and national Greek endemics and secondly to other Important Plant Species (IPS) found in Greece, such as local Balkan endemics or other rare and/or protected plants. Last but not least, the BBGK also focuses on native plants of socio-economic importance i.e. aromatic and/or medicinal plants (Maloupa et al. 2008). To date, about 1,500 taxa (species and subspecies) or ca. 25% of the Greek flora is currently maintained in accessible ex situ conservation collections (Maloupa et al. 2008) and ca. 40% of the collections belong to IPS (Maloupa and Krigas 2008).

Achillea occulta Constantinidis and Kalpoutzakis (Tracheophyta, Asterales, Asteraceae) is a threatened single-mountain Greek endemic (Fig. 1a) and it seems to have no other close relatives in Greece (Constantinidis and Kalpoutzakis 2005, 2009). It is exclusively known from Mt Koulochera and Komboriza, SE Peloponnese, Greece (Fig. 1b), at altitudes ranging from 700 to 1.126 m. *A. occulta* has been recently characterized as "Vulnerable" (Constantinidis and Kalpoutzakis 2009) based on the criteria of IUCN (2001). The habitats of this local Greek endemic are presumably threatened by fires and overgrazing (Constantinidis and Kalpoutzakis 2009) and they have been included in the site GR2540001 (ORI GIDOVOUNI, CHIONOVOUNI, GAIDOUROVOUNI, KORAKIA, KALOGEROVOUNI, KOULOCHERA KAI PERIOCHI MONEMVASIAS) of the European Natura 2000 Network (http://natura2000.eea.europa.eu/).

Bunn et al. (2011), GSPC (2002) and ESPC (2009), Maunder et al. (2001) and Sarasan et al. (2006) have all stressed the need of increased emphasis on applied research in order to develop propagation and cultivation protocols for threatened plants, as contributions to species recovery activities. Re-enforcement of wild plant populations using individuals raised ex situ is considered as a valid means in attempts to reduce the risk of extinction of threatened species (Bowes 1999; Bunn et al. 2011). The in vitro culture is a useful tool in the ex situ conservation of rare, endemic, and threatened plant species with increasing importance to date (Bunn et al. 2007, 2011; Corral et al. 2010; Mallón et al. 2010, 2011; Wadl et al. 2011; Piovan et al. 2010; Sarasan et al. 2006); however, it may not safeguard alone the survival of a plant species in the wild.

The in vitro culture work of threatened plant species is often associated with two inherent problems. First of all, there is frequently a lack of published methods for the species in question and secondly, there is often a limited amount of experimental plant material initially available (Bunn et al. 2011; Krogstrup et al. 2005). Major bibliographic databases such as Scopus or the Web of Science, unfortunately furnish no scientific data concerning the propagation or the conservation of *A. occulta.* Due to the absence of alternative propagation means for this species, its cultivation in the BBGK may offer a potential for future re-enforcement of wild populations, in case the limited wild populations decline or its original habitat is altered and/or destructed due to human activities and/or global warming.

The scope of this study was (a) the development of a rapid, simple, reliable and effective in vitro propagation protocol for *A. occulta* as a contribution to the implementation of Target 8 of the ESPC (2009) and the GSPC (2002), and (b) the development of specific guidelines for its ex situ cultivation in man-made habitats like botanic gardens. To facilitate this scope, the in situ observations and collection data of *A. occulta* have been linked with GIS (Geographical Information Systems) derived-geodata regarding the original wild habitat of this species, following the methodology developed by Krigas et al. (2010). To

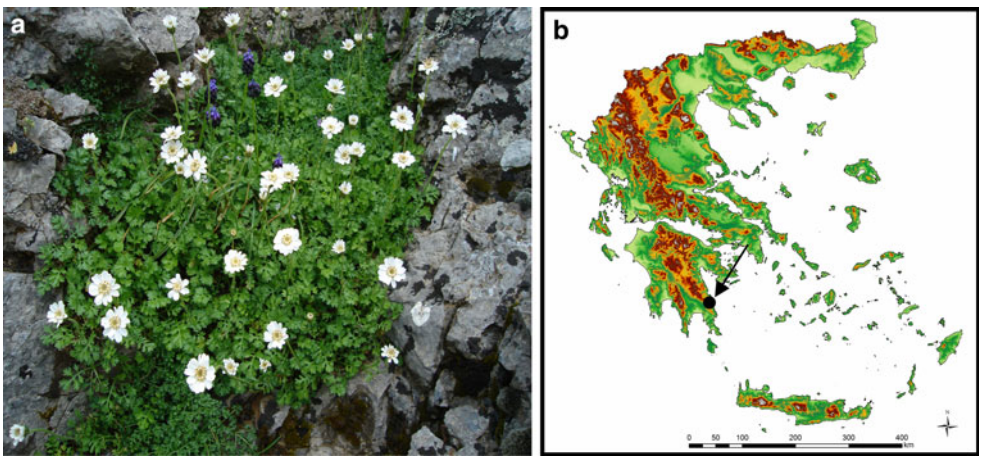

Fig. 1 **a** *Achillea occulta* in its natural habitat: a vulnerable local endemic of Mt Koulochera and Komboriza, SE Peloponnese, S Greece (Photo: E. Kalpoutzakis, with permission) and **b** *Black dot* total known distribution of *A. occulta* in Greece

our knowledge the application of GIS in order to serve the in vitro propagation of a conservation priority species has never been used before.

Materials and methods

Plant material

According to the BBGK's conservation policy *A. occulta*, as a local Greek endemic of narrow distribution, belongs to Priority 1 IPS (Maloupa et al. 2008). A botanical expedition was organized in December 2004 in order to collect plant material of *A. occulta* from the wild, using a special permit issued yearly by the Greek Ministry of Rural Development and Foods (renewed yearly). The plant material collected from the wild has been fully documented (location data, geographical coordinates, habitat information) and obtained an accession number (GR-BBGK-1-04,2640) which follows the numbering of the International Plant Exchange Network (IPEN, http://www.bgci.org/resources/ipen/). Due to the limited population of *A. occulta* recorded in the wild (Constantinidis and Kalpoutzakis 2005), only two (2) wild growing individuals were collected from rocky outcrops in Mt Koulochera, Peloponnese (S Greece), at an altitude of ca. 1.000–1.100 m.

The plants of this species are ecologically specialized to thrive at the rocky slopes and cliffs of the northeastern and eastern summits of the mountain (the only places that locally retain some remnants of tall scrub or tree vegetation), preferring shady or semi-shady rocky places and protruding faces which are protected from blazing sun by the woody vegetation. In its wild habitat, *A. occulta* presents a scattered distribution and it may be found together with some woody plants (Greek endemics marked with an asterisk) such as *Acer sempervirens* L., **Amelanchier parviflora* Boiss. subsp. *chelmea* (Halácsy) Ziel., *Fraxinus ornus* L. subsp. *ornus* and *Phillyrea latifolia* L. and **Rhamnus sibthorpiana* Schult. It grows together with other uncommon herbaceous plants such as **Consolida tuntasiana* (Halácsy) Soó, **Cerastium illyricum* Ard. subsp. *brachiatum* (Lonsing) Jalas, *Crepis fraasii* Sch. Bip., **Cymbalaria microcalyx* (Boiss.) Wettst. subsp. *microcalyx*, **Minuartia pichleri* (Boiss.) Maire and Petitm., **Melica rectiflora* Boiss. and Heldr., **Stachys chrysantha* Boiss. and Heldr., *Thalictrum orientale* Boiss., etc. *A. occulta* may be gregarious in protected rock cracks, where fertile soil is usually accumulated (Constantinidis and Kalpoutzakis 2005).

GIS application and cultivation of mother plants

The geographical coordinates of the original collection site of *A. occulta* were imported into the GIS application (Krigas et al. 2010), and were linked accordingly with numerous digital geodatabases which furnish topographic, climatic (mean values for the last 50 years), soil and habitat data for the same spot; all this information was summarized into an ecological fact sheet for *A. occulta* (Fig. 4).

In an attempt to minimize the transplanting shock and to emulate as best as possible the natural temperatures prevailing in its wild habitat during winter and spring, the initial mother plants have been maintained at 1.5 l pots in a greenhouse of BBGK until next spring with temperatures ranging from $T_{min} > 8°C$ to $T_{max} < 15°C$.

The mother plants collected from the wild were initially propagated by root division during spring 2005 at a rate of 2, producing two (2) individuals from a single root, thus in total four (4) new mother plants.

The initially propagated mother plants have been transferred during summer at an open nursery at sea level with temperatures approaching the natural ones (mean minimum temperatures of 14.7–16.7°C and mean maximum temperatures of 28–30.7°C, Fig. 4).

GIS application and in vitro culture conditions

By the end of spring 2005, the newly formed shoot tips of the initially propagated mother plants of *A. occulta* were collected, stripped of leaves, washed with tap water and disinfected using 1.7% (v/v) NaOCl solution with three drops of Tween 20/500 ml, shaken on a rotary shaker for 10 min and rinsed 3 times with sterile distilled water. After disinfection the external leaves of explants were removed. Under a stereoscope, the internal apex was isolated and inoculated in vitro in test tubes (100 × 20 mm) containing 10 ml of Murashige and Skoog basic medium (MS, Murashige and Skoog 1962) and Woody Plant Medium (WPM, Lloyd and McCown 1980) media supplemented with 2 μM 6-benzyladenine (BA), 0.05 μM indole-3-butyric acid (IBA), 20 g l^{-1} sucrose and 6 g l^{-1} agar (Evenor and Reuveni 2004; Wawrosch et al. 1994). The pH was adjusted to 5.8 prior to agar addition and autoclaving.

In an attempt to emulate as best as possible the natural temperatures prevailing at the wild habitat of the species, the in vitro cultures were kept at 22 ± 2°C day temperature and 15 ± 2°C night temperature, at 16-h photoperiod under cool white fluorescent light (40 μmol m^{-2} s^{-1}).

Explants that had produced adventitious shoots were divided in small shoot clusters and they were transferred into 250 ml Magenta glass vessels containing 35 ml of the same medium. Every 3 weeks the procedure was repeated. After 3 subcultures, the amount of Fe at the basal medium MS was doubled by the addition of 30 mg l^{-1} NaFeEDTA (modMS, Molassiotis et al. 2003). Finally, 11 months later, adequate enough plant material was produced for further in vitro experimentation.

Mineral nutrient medium

Three basic nutrient media, modMS (MS containing double the amount of Fe), WPM and B5 (Gamborg et al. 1968) were tested for the production of adventitious shoots. All media were supplemented with 10 μM BA, 30 g l^{-1} sucrose and 8 g l^{-1} agar (Evenor and Reuveni 2004; Wawrosch et al. 1994).

Effect of cytokinin and auxin combinations

Moreover, 5 and 10 μM BA was combined with 2.5 μM α-naphthaleneacetic acid (NAA) or 2.5 μM IBA to investigate possible different effects of cytokinin/auxin combination on in vitro production of adventitious shoots. The basal media used was the modMS supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar (Evenor and Reuveni 2004; Zoberi et al. 2003).

In vitro rooting and acclimatization

In order to evaluate the root induction ability of the in vitro produced plant material, microshoots produced at the stock cultures were transferred to modMS medium supplemented with 0–20 μM IBA and 0–20 NAA. All rooting media were also supplemented with 30 g l-1 sucrose and 6 g l-1 agar. The cultures were maintained under the same environmental conditions with those used for the initially propagated stock material.

Rooted and non-rooted shoots were rinsed with tap water to remove the adhering medium and then planted separately in trays filled with a peat (Klasmann, KTS 1)-perlite 4:1(v/v) mixture. The trays were placed in a greenhouse under a 90% RH fog-system and 50% shading for 10 days. For the next 10 days, RH was reduced (5%/day), while light intensity was gradually increased per day (Makunga et al. 2003; Wawrosch et al. 1994).

As *A. occulta* occurs in the wild only at altitudes ranging from 700 to 1.126 m (Constantinidis and Kalpoutzakis 2009), only half of the young plants produced were maintained at the nursery of BBGK at sea level. The rest of them were planted at the BBGK's main ex situ conservation area in Pontokerassia (Kilkis Prefecture, N Greece) at an altitude of ca. 600 m which is more close to the natural altitudinal range of *A. occulta*.

Experimental design and statistical analysis

A total of 26 shoot tips from the two parent plants were used for culture initiation. Non contaminated ones were subcultured for 13 times for the production of enough plant material for experimentation.

Shoot tips (1 cm long) from the stock cultures were used as explants. The shoot proliferation experiments lasted for 3 weeks, under the same cultural conditions with those used for the initially propagated stock material. At the end of the cultivation period, the numbers of new microshoots/explant and microshoot height were recorded.

The rooting experiments lasted for 4 weeks; at the end of that period the number of rooted microshoots, number of roots/microshoot and root lengths were measured. Rooting was expressed as percentage (%). The number of successfully acclimatized plants was recorded 2 months after transplanting in the glasshouse and was expressed as survival percentage (%).

The experiments followed a randomized complete block design, with 5 explants per treatment and 8 replications. All experiments were repeated twice. Analysis of variance was performed with the General Linear Model procedure (SPSS 14.0 statistical package) and mean separation with Duncan's Multiple Range Test. Significance was recorded at $P \leq 0.05$ (Steel et al. 1997).

Results

Development of mother plants and GIS-derived data

The GIS derived ecological factsheet of *A. occulta* indicated that during the winter months (December, January, February) this species experiences in the wild mean minimum temperatures of 2.1–3.9°C and mean maximum temperatures of 8–9.7°C. Similarly, during the spring months (March, April, May) it is adapted to mean minimum temperatures of 3.4–9.7°C and mean maximum temperatures of 10.5–19.4°C, while during the summer months (June, July, August) it is adapted to mean minimum temperatures of 13.4–15.8°C and mean maximum temperatures of 24.2–26.3°C (Fig. 4). After balancing out the temperatures of its natural habitat (Fig. 4) with those prevailing at the man-made habitat of the sea level nursery of BBGK (mean minimum temperatures of 10.8–14.7°C and mean maximum temperatures of 23–28°C), the number of initially collected plants was successfully increased to four (4) by root division (spring 2005). When summer temperature increased in late June ($T_{min} > 25$°C and $T_{max} > 35$°C) these mother plants started to decline and finally did not manage to survive. Only plant material established in vitro remained alive.

GIS-derived data and in vitro culture conditions

Disinfection procedure of explants was quite satisfactory and only 23% of them were contaminated by bacteria,

whereas 3.8% were not developed. As indicated by the species' ecological fact sheet (Fig. 4), the natural temperatures prevailing in the wild during May and June (when the active growth occurs) may range from 9.7–13.4°C to 19.4–24.2°C (Fig. 4) dictating the selection of 22 ± 2°C day temperature and 15 ± 2°C night temperature, at 16-h photoperiod, to be used as most suitable for the development of the in vitro cultures.

Explants established in MS medium developed comparatively faster and presented better physiological appearance (Fig. 2a). On the contrary, explants in WPM presented a slow development and their leaves started to turn yellow and showed peripheral necrotic spots. All non-contaminated explants were transferred to MS basal medium but after 3 subcultures leave yellowness appeared. After doubling the amount of Fe, the growth of the stock cultures was slow but stable (Fig. 2b).

Basal nutrient medium

WPM resulted in significantly increased number of new microshoots produced/explant (4.05, 1.02 cm long) but 57% appeared necrotic leaves, shoot tip stopped to increase and finally new microshoots were destroyed (Table 1; Fig. 2c). The number of new microshoots/explant in MS medium was smaller (2.48, 1.00 cm long) but only the 34% presented the phenomenon of necrosis. New microshoots were not developed in B5 medium and at the end of subculture period existed leaves were malformed and turn in a yellow–brown color (Table 1; Fig. 2c).

Table 1 Effect of basal culture media on in vitro adventitious shoot production of *A. occulta*

Media	No. of new shoots/explant	Shoot height (cm)	Percentage of destroyed shoots (%)
modMS	2.48 b	1.00 a	34 a
WPM	4.05 a	1.02 a	57 b
B5	0 c	0 b	100 c

modMS = MS NaFeEDTA 30 mg l^{-1}, all media were supplemented with 10 μM BA, 30 g l^{-1} sucrose, 8 g l^{-1} agar, pH 5.8

a, b, c: Different letters within columns indicate significant differences according to the Duncan's range test ($P \leq 0.05$). Data represent the mean from two replications of each experiment. Shoot tips 1 cm long were used as explants

Effect of cytokinin and auxins combinations

Even though the addition of IBA or NAA in combination with BA did not seem to have significant effect neither on the proliferation rate nor at the shoot height (Table 2), the presence of IBA at 2.5 μM IBA resulted in better physiological appearance and absence of leaf necrosis

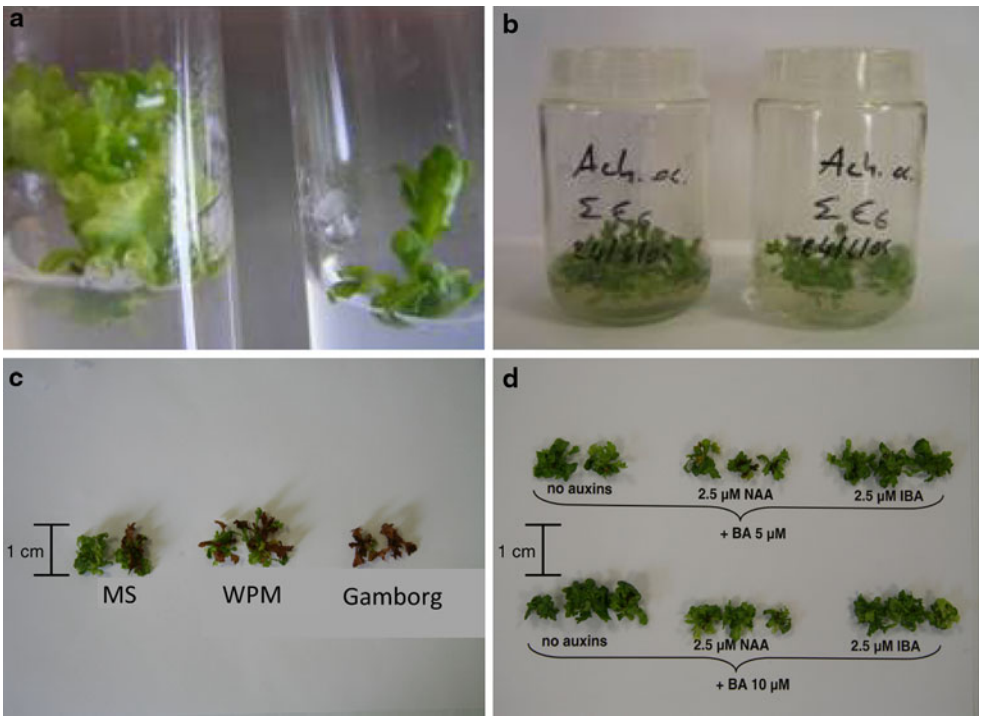

Fig. 2 **a** Establishment of *A. occulta* in vitro in Murashige and Skoog (1962) medium (*left*) and Woody Plant Medium (Lloyd and McCown 1980) (*right*), **b** stock in vitro cultures of *A. occulta* in modified Murashige and Skoog (1962) medium (MS + NaFeEDTA 30 mg l^{-1}), **c** effect of basal culture media on in vitro adventitious shoot production of *A. occulta* and **d** effect of BA or BA + IBA and NAA on in vitro adventitious shoots of *A. occulta*

Table 2 Effect of BA in combination with NAA or IBA on in vitro adventitious shoots of *A. occulta*

Media	No. of new shoots/explant	Shoot height (cm)
BA 5 μM	3.4 a	1.00 a
BA 5 μM + NAA 2.5 μM	2.1 b	0.89 b
BA 5 μM + IBA 2.5 μM	3.5 a	0.92 ab
BA 10 μM	3.6 a	0.93 ab
BA 10 μM + NAA 2.5 μM	2.9 a	0.89 b
BA 10 μM + IBA 2.5 μM	3.2 a	0.84 b

Basic media used was modMS (MS + NaFeEDTA 30 mg l^{-1})

a, b: Different letters within columns indicate significant differences according to the Duncan's range test ($P \leq 0.05$). Data represent the mean from two replications of each experiment. Shoot tips 1 cm long were used as explants

(Fig. 2d). The combination of 5 μM BA with 2.5 μM IBA presented a high number of new microshoots/explant (3.5), with satisfactory shoot height (0.93 cm) (Table 2; Fig. 2d).

In vitro rooting and acclimatization

Rooting proved to be very difficult and only by adding 20 μM IBA, a 12.5% rooting percentage was achieved (Table 3). Microshoots cultured at rooting media appeared yellow-colored and declined. These side effects were more intense at high concentrations of NAA (10 and 20 μM).

The acclimatization procedure was successful when rooted plantlets were planted in the greenhouse only during early spring. The selection of the appropriate transplanting period was made by combining the temperature profile of the natural habitat of *A. occulta* indicated by the GIS application (Fig. 4), the natural growth period of the species in consideration (early spring, Constatinidis and Kalpoutzakis 2009) and the temperature profile of the BBGK's nursery during early spring (almost similar to that of the natural habitat of *A. occulta*).

Furthermore, some plants which received the auxin effect (media with 10 and 20 μM IBA and 2.5 μM NAA) formed roots in vivo during acclimatization and finally the survival percentage was increased compared to the rooting percentage (Table 3; Fig 3a). After 2 months, the plants were developed enough to be transplanted in bigger pots for further development.

Ex situ conservation and GIS-derived data

In an attempt to emulate the natural substrate as indicated by the GIS application (Fig. 4), the in vitro produced plants were transplanted in a mixture of peat (Klasmann, TS 2) and vermiculite (2:1) resulting in a coarse texture close to the natural substrate. In this way, the in vitro produced plants continued developing without problems in a shady (50%) place at the nursery of BBGK (sea level) during spring.

The plants that remained at the at sea level nursery (10 individuals) started to decline when summer temperature increased ($T_{min} > 25°C$ and $T_{max} > 35°C$) and finally only one individual remained alive till next autumn (10% survival). On the contrary, all of the 10 young plants that were transplanted at the BBGK (600 m) have successfully adapted and produced flowers and seeds from the first year (Fig. 3b). The BBGK's main ex situ conservation area is characterized by a temperature profile with mean winter temperatures T_{min} −1.9 to −0.1°C and T_{max} 5–7.4°C (comparatively lower than those of the natural habitat of *A. occulta*, Fig. 4), mean spring temperatures T_{min} 1.7–10°C and T_{max} 11.1–21.5°C (almost similar to those of the natural habitat of *A. occulta*, Fig. 4), mean summer temperatures T_{min} 13.7–15.9°C and T_{max} 25.9–28.7°C (almost similar to those of the natural habitat of *A. occulta*, Fig. 4),

Table 3 Effect of IBA and NAA on in vitro rooting and acclimatization of *A. occulta*

Media	Rooting (%)	No. of roots/micro shoot	Root length (cm)	Successfully acclimatized plants (%)
0 μM IBA	0 c	0 b	0 c	0 d
2.5 μM IBA	0 c	0 b	0 c	0 d
5 μM IBA	0 c	0 b	0 c	8 c
10 μM IBA	8 b	1.5 a	0.63 b	22.5 b
20 μM IBA	12.5 a	2 a	0.93 a	33 a
2.5 μM NAA	0 c	0 b	0 c	3 cd
5 μM NAA	0 c	0 b	0 c	0 d
10 μM NAA	0 c	0 b	0 c	0 d
20 μM NAA	0 c	0 b	0 c	0 d

Basic media used was modMS (MS + NaFeEDTA 30 mg l^{-1})

a, b, c: Different letters within columns indicate significant differences according to the Duncan's range test ($P \leq 0.05$). Data represent the mean from two replications of each experiment. Shoot tips 1 cm long were used as explants

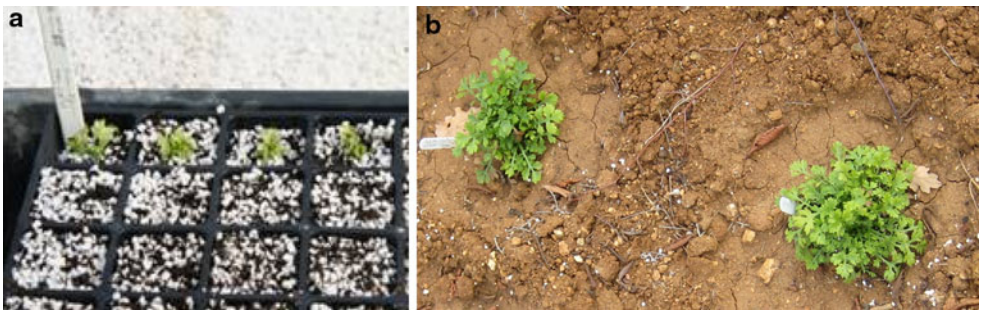

Fig. 3 **a** Acclimatization of in vitro produced plants of *A. occulta* and **b** in vitro produced plant material of *A. occulta* established in the Balkan Botanic Garden of Kroussia, N Greece, at 600 m of altitude

and mean autumn temperatures T_{min} 3.8–12.5°C and T_{max} 11.7–24.6°C (almost similar to those of the natural habitat of *A. occulta*, see Fig. 4).

Discussion

This study reports, in full and for the first time, a GIS-facilitated methodological procedure for the in vitro culture and ex situ cultivation of *A. occulta,* a rare threatened plant which is restricted to a single mountaintop in Peloponnese, S Greece. This could be considered as a contribution towards the implementation of Target 8 of the ESPC (2009) and the GSPC (2002).

The GIS application used have provided a reliable quantitative and qualitative description of the natural habitat of *A. occulta* (Fig. 4) which is ecologically meaningful and can be exploited for the in vitro propagation and the ex situ conservation of this threatened species (Constantinidis and Kalpoutzakis 2009). The propagation trials presented in this study were based rather on ecological criteria than on horticultural experience exclusively. In this way, the usual trial-and-error losses of plant material during the propagation and cultivation procedures in botanic gardens have been obviously avoided. This seems to be significant if taken into account (a) the absence of previously published propagation methods for the species in question, and (b) the restricted amount of valuable plant material initially available for experimentation, due to limited population size of *A. occulta* in the wild (Constantinids and Kalpoutzakis 2009). The methodology applied in this study has addressed productively the above mentioned inherent difficulties related to the in vitro propagation of rare and threatened species (Bunn et al. 2011; Krogstrup et al. 2005).

The GIS application used in the present study has contributed to the selection of the appropriate: (a) temperatures for both greenhouse cultivation and in vitro cultures, (b) period for acclimatization and transplanting of plantlets produced in vitro, (c) growing media and substrates, (d) ex situ conservation sites, and (e) spatial and temporal positioning within the nurseries and the ex situ conservation sites of the BBGK.

The preferences of *A. occulta* for rather mild temperatures (between 2.1°C during the coldest month and 26.7°C during the hottest month, Fig. 4) have explained the mother plants losses during the hot summer period. The weakness or death of the initially propagated stock plants due to heat stress and high summer temperatures have also been reported for other members of Asteraceae family (Evenor and Reuveni 2004; Zoberi et al. 2003).

The loss of the mother plants originating from the natural environment due to heat stress as suspected from other studies in members of Asteraceae family (Evenor and Reuveni 2004) has actually confirmed the choice of the in vitro propagation as the most appropriate method. For a long time, the in vitro established plant material was the only possibility to produce new young plants. The GIS application facilitated the selection of day and night temperatures for the in vitro culture, dictating a relative low night temperature (15°C ± 2) to be selected for the tissue culture conditions (Fig. 4).

Different culture media have been used for in vitro culture of other members of the family of Astreaceae (Evenor and Reuveni 2004; Mallón et al. 2011; Wawrosch et al. 1994) with the MS basal medium being the most applied one. Shoot production of *A. occulta* was enhanced when shoot tip explants were cultured in MS medium, which proved to be the most adequate among the media tested. Even though WPM has a very good balance of macro-elements, presented inferior results causing yellowness and necrotic leaves, probably due to the lower concentrations. MS medium is a strong culture medium containing 3 to 4 times more the amounts of macro-elements compared to the other media tested (Murashige and Skoog 1962). The uptake, transport, or requirement of Fe and other nutrients at tissue culture systems resulting in reduced chlorophyll formation and chlorosis, depends upon the equilibrium of a series of factors like genotype, temperature, pH and growth regulators, among others

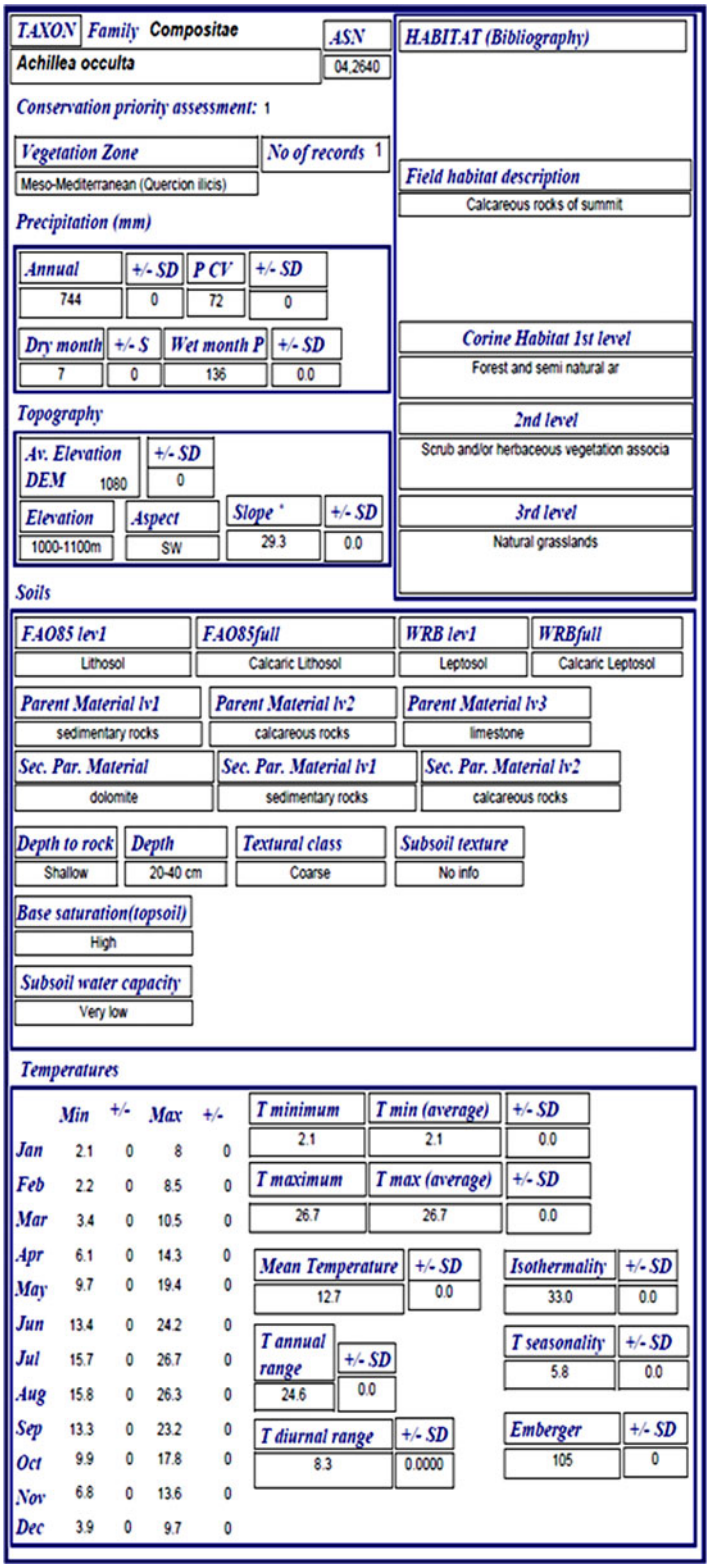

Fig. 4 Ecological factsheet of *A. occulta* derived from Geographical Information Systems geodata concerning its natural habitat (average values for the last 50 years). For the methodology used see Krigas et al. (2010)

(Dolcet-Sanjuan et al. 1990; Monteiro et al. 2000). The MS medium contains Fe in the chelate form of Fe-EDTA, although of all chelates known the most effective form is Fe-EDDHA (Molassiotis et al. 2003). The demand of twice the amount of Fe used for the *A. occulta* tissue culture could be explained due to the lower cultivation temperatures used for the tissue cultures, in which Fe becomes less exchangeable and available to plants and to the chelate

form of Fe at MS basic culture media. Possible substitution with Fe-EDDHA could provide better results (Molassiotis et al. 2003).

The addition of 5 μM BA plus 2.5 μM IBA at the basal culture medium proved to be the most effective combination of growth regulators. By increasing the BA over 2.5 μM, no significant differences were noticed, thus this concentration was chosen as the most appropriate. Furthermore the use of auxin proved to be critical. Only when an auxin was present, leave malformations disappeared (Fig. 2c, d). The same response has been reported for *Achillea asplenifolia* (Wawrosch et al. 1994). Among the two auxins tested, IBA resulted in higher proliferation rate and shoot height. It seems that an IBA to BA ratio about 0.5 was needed for proper microshoot production in *A. occulta*. These results agree with reports for other members of Astreaceae family demanding an auxin to cytocinin ratio between 0.3 and 0.5 (Evenor and Reuveni 2004).

Rooting in vitro was the critical point for successful acclimatization of young plantlets. The total number of rooted in vitro plants managed to survive and acclimatized. Rooting and acclimatization of other Asteraceae members have been reported without problems, with a success ranging between 60 and 100% (Evenor and Reuveni 2004; Wawrosch et al. 1994). The in vitro rooting of several Asteraceae species usually demands a rather small amount of auxins. On the contrary, for *A. occulta* only high IBA concentration has led to the limited in vitro rooting presented in this study. The low rooting ability and rather difficult acclimatization of this species could be connected with its ecological specialization (Constantinidis and Kalpoutzakis 2009). The application of alternative in vitro techniques for enhanced rooting (such as combination of different auxins in various concentrations, addition of activated charcoal in the media or use of ½ MS or ¼ MS basal medium) used for other endangered plants (Guohua et al. 2011a, b, Gonçalves et al. 2011; Irvani et al. 2010), may also prove to be beneficial for *A. occulta* as well.

To date, ex vitro transfer of in vitro produced plants has been based on the use of a range of commercially available substrates, although it has been emphasized that more consideration must be given to the environment-specific needs of the plant in question (Benson et al. 2000). The field observations and the GIS application indicated that *A. occulta* demands a rather fresh climate and is adapted to thrive in relative dry calcaric lithosols (Fig. 4). The type of commercial peat and the addition of vermiculite often used for further development of young plants seem to provide a very good imitation of the natural soil conditions of *A. occulta*; this possibly explains why plantlets continued growing without problems till summer temperature was considerably increased.

In the specific area of BBGK (600 m altitude) selected for the ex situ conservation of *A. occulta*, the mean summer day temperatures are comparatively lower than those at the sea level nurseries and the mean summer night temperatures are noticeably lower; this seems to match better with the temperatures prevailing at the species' natural habitat (Fig. 4). Although in the selected area T_{min} is often below zero during winter, this did not seem to harm the survival of the established young plants of *A. occulta* which have continued developing, flowering and forming achenes 2 years after they have been established in BBGK.

Prior to planting at the BBGK, the basic ecological profile of *A. occulta* was consulted in order to emulate accordingly the natural conditions in a man-made habitat (the conservation area of a botanic garden). The GIS-derived ecological profile of *A. occulta* (Fig. 4) dictated (semi-) shady limestone cracks and rock bases with calcaric lithosols and a natural positioning at S, SE and SW exposures of rock formations (both also confirmed by in situ observations), a natural water amount received equal to a mean total annual precipitation of 744 mm (7–136 mm per month), and a temperature range equal to 2.1–26.7°C, with a mean diurnal range of 8.3°C. All these assets were followed or emulated as best as possible at the grounds of the main ex situ conservation area of BBGK.

Currently a small population of *A. occulta's* propagated plants is maintained ex situ in the BBGK where plants flower and produce fruits with no problems observed so far (seeds are regularly collected for future experimental studies). With the aim to obtain good representation of the species' genetic diversity for an effective ex situ conservation, another botanic expedition is scheduled for next autumn in order to collect seeds from numerous wild growing individuals of *A. occulta* to be used for GIS-facilitated germination studies.

Among others, Bunn et al. (2011) and Maunder et al. (2001) have prioritized the need of applied research to develop propagation and cultivation protocols for threatened plants, as contributions to future species recovery activities, while ESPC (2009) and GSPC (2002) have included this urgent need under Target 8 in their strategic plans for plant conservation. The ecological preferences of *A. occulta* in the wild that were successfully revealed with the use of the GIS application developed by Krigas et al. (2010), may offer an example of how effective bridges between in situ and ex situ conservation may be constructed. As presented in this study, this effective linkage may (a) facilitate the in vitro propagation and initial cultivation of valuable plant material belonging to conservation priority species and (b) may also provide several ecologically based guidelines for ex situ conservation of target species threatened with extinction.

Acknowledgments The authors would like to thank Assist. Prof. Th. Constantinidis for offering the initial plant material of *A. occulta* to the Balkan Botanic Garden of Kroussia, as well as the two anonymous reviewers for helpful comments on the first version of the manuscript.

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