International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 27-09-2020; Accepted: 13-10-2020: Published: 28-10-2020
www.botanyjournals.com
Volume 5; Issue 5; 2020; Page No. 532-539

In vitro propagation of *Primula veris* L. subsp. *veris* (Primulaceae): A valuable medicinal plant with ornamental potential

Katerina Grigoriadou[1*], Virginia Sarropoulou[2], Nikos Krigas[3], Eleni Maloupa[4]
[1-4] Hellenic Agricultural Organization (HAO)-DEMETER, Institute of Plant Breeding and Genetic Resources, Thessaloniki, Greece

Abstract
The native populations of *Primula veris* L. subsp. *veris* in Greece are often harvested directly from the wild due to known medicinal properties and ornamental value and the plant material is sold in national and international markets. Therefore, *ex-situ* conservation efforts are urgently needed for its sustainable use. The *in vitro* effect of gibberellic acid GA_3 (three ways of application), light (16h photoperiod, 24h dark) and storage time (17, 14, 8 years, respectively, stored in 20 ^oC and relative humidity <5%) on seed germination of *P. veris* subsp. *veris* was investigated. MS basal medium enriched with 20 g/L sucrose and 6 g/L agar was used. After 60 days, the germination rate reached 50% when 250 mg/L GA_3 was added in the medium. Light did not affect the seed germination ability; however, growth after germination was faster with high quality seedlings. No germination was detected in seeds stored for 17 and 14 years, while those stored for 8 years germinated at about 50%. The effect of GA_3 was critical, since the trials with no GA_3 presented <7% germination rate or no germination at all. Storing time of >10 years affected negatively the seed germination; therefore, their renewal is necessary in seed banks at least every decade. The use of GA_3 favours the onset of germination. In the subsequent shoot proliferation stage, the MS medium enriched with either 4.4 or 6.6 μM BA in combination with 0.1 μM IBA and 0.3 μM GA_3 gave optimum results within a three-week period in terms of shoot formation (100%), shoot number (3.2-3.7 shoots/ explant) and shoot proliferation rate (1.6-2.2) at the same time. All BA concentrations led to the production of multiple shoots in 100% of the explants, whereas no shoot formation was observed in the control treatment (BA-free). In the following rooting stage, IBA exhibited 100% rooting irrespective of applied concentration (2.5 and 5 μM), and caused a 2.5-3-fold increase in root number (5.7-6.9 roots/rooted explant) compared to IBA-untreated explants. However, root length was greater (1.94 cm) in the case of the control, as both IBA concentrations had an inhibitory effect. The *ex vitro* acclimatization of rooted microplants was successfully completed (100% survival) within 26 days in the mist in a peat: perlite (1:1) mixture. Therefore, an efficient micropropagation protocol for *P. veris* subsp. *veris* was developed from *in vitro* germinated seeds as starting material.

Keywords: 6-benzyladenine, *ex situ* conservation, indole-3-butyric acid, medicinal plants, plant growth regulators, cowslip

Introduction
Primula spp. (primulas, cowslips, oxlips, auriculas and primroses, Primulaceae) are popular garden ornamentals but also wild-growing plants. In Greece, there are many commercial hybrids widely introduced as cultivated ornamentals for home gardening as well as some wild-growing species and subspecies, i.e. *P. intricata* Gren. & Godr. (non-confirmed occurrence in Greece), *P. elatior* (L.) Hill and *P. veris* L. subsp. *veris* which occur naturally at intermediate and higher altitudes in woodlands, scrub and forest edges of the mainland (the latter also in the island of Samothraki), and *P. vulgaris* Huds. (the common European subsp. *vulgaris*, and the subsp. *rubra* with Balkan-Anatolian range) occurring both in parts of the mainland and some of the Greek islands (Dimopoulos *et al.*, 2013) [1]. The medicinal potential of *P. veris* and *P. elatior* is currently supported by a finalised Monograph of the European Medicines Agency - Committee on Herbal Medicinal Products (Länger, 2012; https://www.ema.europa.eu/en/documents/herbal-monograph/final-community-herbal-monograph-primula-veris-l/primula-elatior-l-hill-flos_en.pdf) [2] in which whole flower preparations (herbal remedies, or extracts) have specific indications for treating cough, respiratory problems, expectorant cough and bronchitis and are also associated with diuretic, antioxidant, anticonvulsant and anti-inflammatory properties. To date there has been a great commercial demand for dried plant material of *Primula* spp. which probably triggers the uncontrolled over-harvesting of wild plant populations, even in illegal ways. During the last two years, large quantities of hundreds of kilograms of illicit cowslip collections (*P. veris* subsp. *veris*) have been seized repeatedly by the Greek authorities in the region of Epirus (north-west Greece) since they were intended to be exported illegally to Albania and from there to be trafficked (https://www.thenationalherald.com/198540/greek-police-seize-horses-illegally-harvested-wild-herbs/). Therefore, there is an urgent need to protect the extant wild populations from uncontrolled collections on one hand and to facilitate the cultivation of *Primula* spp. on the other. The introduction of *Primula* spp. in farming systems close to their natural habitats may prove to be an alternative to increase agricultural income, and may motivate young people towards alternative crops of high demand that are naturally adapted to local conditions.

Primula spp. are probably not the easiest plants to propagate and cultivate effectively, although there are many popular garden hybrids produced sexually. Seed germination has been reported to be difficult (Coumans *et al.*, 1979; Draxler *et al.*, 2002; Morozowska, 2002) [3-5] while propagation by cuttings cannot be realized due to the morphology of the plants, i.e. dense rosette of leaves close to the ground. *In vitro* culture and regeneration of plants has been reported for

Primula spp. such as *P. obconica* Hance (Bajaj, 1981; Coumans *et al*., 1979) [3, 6], *P. vulgaris* (Merkle and Götz, 1990) [7], *P. malacoides* Franch. and *P. obconica* (Mizuhiro *et al*., 2001) [8] and several *Primula* hybrids (Borodulina *et al*., 2001) [9]. In micropropagation studies conducted for *P. cuneifolia* Ledeb., *P. scotica* Hook., *P. veris*, *P. latifolia* Lepeyr. (= *P.* x *pubescens* Loisel.), and *P. heterochroma* Stapf new plantlets derived from *in vitro* growth of seedlings (Shimada *et al*., 1997; Benson *et al*., 2000; Morozowska and Wesolowska, 2004; Takihira *et al*., 2007; Hamidoghli *et al*., 2011) [10-14] and different cytokinin types were used for shoot production and root formation (Shimada *et al*., 1997; Yamamoto *et al*., 1999; Schween and Schwenkel, 2002, 2003) [10, 15-17]. More recently, adventitious shoot regeneration of *P. latifolia* was reported using TDZ (Takihira *et al*., 2007) [13].

In this framework, the present study reports an optimized protocol for the micropropagation of *Primula veris* subsp. *veris* via shoot tip explants derived from seedlings. Furthermore, seed germination after long storage in seed banks on *in vitro* conditions was also investigated in order to facilitate the mass production of plants and the sustainable cultivation of *P. veris* subsp. *veris*.

2. Materials and methods

2.1 Plant material

Botanical expeditions were organized in spring of 2001, 2004 and 2010 in Northern Greece (Mt Falakro for the first two and Mt Pangaio) in order to collect living plant material and/or seeds of *P. veris* subsp. *veris* from wild-growing populations at high-mountain altitudes (1780-1850 m above sea level), using a special permit issued yearly by the Greek Ministry of Environment and Energy (renewed every year). The collected plant materials were transferred at the facilities of the Balkan Botanic Garden of Kroussia (BBGK) and received IPEN (International Plant Exchange Network) accession numbers, i.e. GR-1-BBGK-01,1460, GR-1-BBGK-04,2603, GR-1-BBGK-10,5428, respectively. For their desiccation, the seeds were maintained for 30 days in a dark chamber at 15 ºC and relative humidity (RH) 15%. For long-term storage (8-17 years), the seeds were stored in sealed containers within a seed bank at 4ºC and RH <5%. The experimentation started in summer of 2018 using stored seeds for 17, 14 and 8 years.

2.2 In vitro seed germination and establishment of culture

The *in vitro* germination of seeds in dark or light after prolonged storage was studied, examining also the effect of gibberellic acid (GA_3) applied in three ways (addition into the media, pre-treatment of seeds by immersion in 250 mg/L GA_3 solution for 20 min, and combination thereof). MS (Murashige and Skoog, 1962) [18] culture medium was used supplemented with 20 g/L sucrose and 6 g/L agar. The pH was adjusted to 5.8 prior to addition of the gelling agent and autoclaving at 121°C for 20 min. Seeds were disinfected using Signum fungicide (0.070g/100ml H_2O) stirred for 30 min in 30 ml of solution, followed by 3% sodium hypochlorite (NaOCl) for 15min and five rinses with sterile dH_2O. The seeds were placed under aseptic conditions inside a laminar flow hood in Magenta vessels (200 ml volume) containing 25 mL of media, and were transferred to a growth chamber at 22 ± 2ºC adjusted to 16 h photoperiod or they were placed in total darkness (24 h).

Eight different treatments were tested, half under 16h photoperiod (40 µmol m^{-2} s^{-1}) [treatment 1: MS medium; 2: MS medium and pre-treatment in 250 mg/L GA_3 for 20 min; 3: MS medium supplemented with 250 mg/L GA_3; 4: MS medium supplemented with 250 mg/L GA_3 and pre-treatment in 250 mg/L GA_3 for 20 min], and half in dark [treatment 5: MS medium; 6: MS medium and pre-treatment in 250 mg/L GA_3 for 20 min; 7: MS medium supplemented with 250 mg/L GA_3; 8: MS medium supplemented with 250 mg/L GA_3 and pre-treatment in 250 mg/L GA_3 for 20 min].

The eight experimental treatments included three (3) vessels/treatment and five (5) seeds/vessel (15 seeds/treatment). Germinated seeds were kept in the vessels for 60 days and afterwards they were transferred in MS basal nutrient medium supplemented with 2 µM 6-benzyladenine (BA), 0.3 µM α-naphthalene acetic acid (NAA), 30 g/L sucrose, and 6 g/L agar (Morozowska and WesŁowska, 2004). Every three weeks, they were sub-cultured at the same medium. The stock material produced was used for further experimentation.

2.2. In vitro culture proliferation and rooting

The exogenous effect of the cytokinin BA applied at four increasing concentrations (0, 2.2, 4.4 and 6.6 µM) on shoot proliferation was tested. The basal medium used was the MS supplemented with 0.5 µM indole-3-butyric acid (IBA), 0.3 µM GA_3, 20 g/L sucrose, and 6 g/L agar, at pH 5.8. After three weeks of culture, shoot formation, shoot number/explant, shoot length (cm), proliferation rate, rooting percentage (%), number of roots/plantlet and root length (cm) were recorded.

Subsequently, in the rooting stage, shoot tip explants 0.5 cm long from stock cultures were used for studying the exogenous effect of the auxin IBA applied at three increasing concentrations (0, 2.5 and 5 µM) on the rooting potential of microshoots. After three weeks of culture, rooting percentage (%), number of roots/rooted microshoot, root length (cm), number of shoots/explant and shoot length (cm) were measured.

In both micropropagation stages (shoot proliferation and rooting), the basal culture medium used for the experiments was the MS fortified with 20 g/L sucrose and solidified with 6 g/L plant agar (Duchefa, The Netherlands). The pH was adjusted to 5.8 before adding the gelling agent and prior to autoclaving at 121°C for 20 min. Explants were transferred into Magenta vessels containing 35 ml of medium. All cultures were maintained in a growth chamber at 22 ± 2°C with a 16 h photoperiod (40 µmol m^{-2} s^{-1}) supplied by cool white fluorescent lamps.

2.3. Acclimatization of *in vitro* produced plantlets

In early March 2019, the rooted microplants obtained from the three IBA treatments with well-developed roots and shoots were washed with tap water and were transferred to a peat moss (Terrahum): perlite (Geoflor) (1:1 v/v) substrate. The plantlets were transferred into multiple – hole propagation trays and were placed on a bench plastic tunnel with adjustable relative humidity 65-72% (internal heated mist system) for 28 days, under non-heated greenhouse conditions. The successfully acclimatized plants were transplanted into larger volume pots (0.33 L – 8x8x7 cm) containing an enriched in organic matter substrate mixture of peat moss (TS2, Klassmann): perlite (Geoflor) (3:1 v/v ratio) and were placed outdoors (external environmental

conditions under shading), and they were irrigated manually when needed. After a three-month period, in early summer, plants were transplanted into pots of 2.5 L containing a peat moss (TS2): perlite substrate (3:1 v/v ratio) and were maintained in external environment under shading, without direct exposure to sun and rain. Irrigation was provided manually when necessary, depending on the environmental conditions. Observations were taken after a period of 26 days in the mist and afterwards at the end of the acclimatization period outside greenhouse (end of August). Data were obtained after six months (i.e. from rooted microplants to fully acclimatized plants, March – August) and their survival percentage was recorded.

2.4 Statistical analysis

In the initial germination stage for seeds the experiment was a 2x2x2 factorial one, i.e. two basic conditions (16 h photoperiod and 24 h darkness), two pre-treatments in GA_3 solution (0 and 20 min) and two culture media fortified with GA_3 (250 mg/L) or not fortified (no GA_3). The main effect of factors and their interactions were determined by the General Linear Model (3-way ANOVA). The experimental layout was completely randomized. Means were subjected to analysis of variance (ANOVA) and were compared using the Duncan multiple-range test ($P<0.05$).

In the shoot proliferation stage, the experiment included four (4) treatments with 10 replicates (2 Magenta vessels x 5 explants each). In the rooting stage, the experiment included three (3) treatments with 16 replicates (4 Magenta vessels x 4 explants each). Both experiments were completely randomized, they were repeated twice, and the results were analysed with ANOVA using the statistical program SPSS 17.0 (SPSS Inc., Illinois, New York, USA) at $P \leq 0.05$, according to Duncan's multiple range test ± SE in order significant differences among treatments to be established.

3. Results

3.1 *In vitro* seed germination and establishment of culture

The seeds collected in 2001 and 2004 (17- and 14-years-old) did not germinate at all, and many of them showed bacterial and fungal infections, whereas those collected in 2010 germinated without signs of pathogens (Fig. 3a-b). After 20 days of culture, the germination rate in light was higher (33.33%) in MS medium fortified with 250 mg/L GA_3 without pretreatment. At the end of the experiment, 60 days after disinfection and initial establishment, the following three treatments (a and b in light; c in dark) exhibited the higher seed germination rate (46.67%): (a) MS medium with 250 mg/L GA_3, pretreatment in 250 mg/L GA_3 - light, (b) MS medium with 250 mg/L GA_3 - light, and (c) MS medium with 250 mg/L GA_3 - dark. No germination was observed when seeds were cultured in plain (non-fortified) MS medium with no pretreatment in dark, and low germination (6.67%) under the 16h-photoperiod regime. Germination started the 20th day after initiation of the culture. When seeds were cultured in MS medium contained 250 mg/L GA_3, there was a progression of the germination process from 20 to 60 days associated with an increase in germination rate in both regimes (16 h light, 24 h dark), irrespective of previous immersion in GA_3 solution as a pretreatment. However, when seeds were cultured in MS medium without GA_3, regardless previous pretreatment, seed germination rate reached its maximum value after 20 days of culture and was remained stable until the 60 days in either light or dark conditions (Table 1).

Table 1: *In vitro* germination of *Primula veris* subsp. *veris* seeds collected in 2010 (8 years-old) from Mt Pangaio (Northern Greece): Number of germinated seeds and germination rate (%) 20 and 60 days from the establishment of *in vitro* culture

Treatments			Number of germinated seeds		Seed germination rate (%)	
			in 20 days	in 60 days	in 20 days	in 60 days
16 h light	No pretreatment	MS	1 c	1 d	6.67 d	6.67 d
	Pretreatment GA_3	MS	4 ab	4 bc	26.67 b	26.67 b
	No pretreatment	MS + GA_3	5 a	7 a	33.33 a	46.67 a
	Pretreatment GA_3	MS + GA_3	3 b	7 a	20.00 c	46.67 a
24 h dark	No pretreatment	MS	0 c	0 d	0.00 d	0.00 d
	Pretreatment GA_3	MS	3 b	3 c	15.00 c	15.00 c
	No pretreatment	MS + GA_3	1 c	7 a	6.67 d	46.67 a
	Pretreatment GA_3	MS + GA_3	1 c	5 b	6.67 d	33.33 b
p-values (3-way ANOVA)						
16 h light / 24 h dark (A)			0.000***	0.014*	0.000***	0.000***
Pretreatment in GA_3 solution (yes/no) (B)			0.006**	0.014*	0.002**	0.008**
MS medium with and without GA_3 (C)			0.132 ns	0.000***	0.006**	0.000***
(A)*(B)			0.132 ns	0.014*	0.174 ns	0.020*
(A)*(C)			0.006**	1.000 ns	0.002**	0.491 ns
(B)*(C)			0.000***	0.000***	0.000***	0.000***
(A)*(B)*(C)			0.132 ns	0.014*	0.006**	0.257 ns

Means denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$; ns $P \geq 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

3.2 *In vitro* culture proliferation and rooting

Shoot formation 100% was observed in all treatments containing BA, irrespective of concentration. No shoot induction was observed in the control BA-untreated explants. Shoot number was maximized when explants were treated with 4.4 µM BA. Shoot length was highest in the control and in 6.6 µM BA, while it was slightly reduced at intermediate concentrations. The highest shoot proliferation rates (1.7-2.2) were obtained by the addition of 4.4-6.6 µM BA into the culture medium. Rooting was reduced with increasing BA concentration, and 6.6 µM BA caused complete inhibition of rooting. The increased BA concentration diminished both the number of roots/ microplant (0.3-1.4) and root length (0.63-0.85 cm) comparing to the control (6.4 roots, 1.85 cm long) (Table 2, Fig. 1).

Table 2: Effect of BA concentration (0, 2.2, 4.4 and 6.6 µM) combined with 0.1 µM IBA and 0.3 µM GA_3 on shoot formation (%), shoot number/explant, shoot length (cm), proliferation rate, rooting (%), number of roots/ microplant and root length (cm) of *Primula veris* subsp. *veris* explants after 3 weeks of culture in MS medium with 20 g/L sucrose and 6 g/L agar

BA (µM)	Shoot formation (%)	Shoot number/ explant	Shoot length (cm)	Proliferation rate	Rooting (%)	Root number/ microplant	Root length (cm)
0	0	1.00 ± 0.00 c	0.50 ± 0.00 a	1.00 ± 0.00 b	100 a	6.40 ± 0.78 a	1.82 ± 0.13 a
2.2	100	2.80 ± 0.20 b	0.42 ± 0.02 b	1.00 ± 0.00 b	70 b	1.40 ± 0.43 b	0.85 ± 0.07 b
4.4	100	3.70 ± 0.40 a	0.40 ± 0.01 b	1.60 ± 0.27 ab	20 c	0.30 ± 0.21 bc	0.63 ± 0.02 c
6.6	100	3.20 ± 0.29 ab	0.51 ± 0.04 a	2.20 ± 0.36 a	0 d	0.00 ± 0.00 c	0.00 ± 0.00 d
p-values	-	0.000***	0.000***	0.001***	0.000***	0.000***	0.000***

Means (n=10) ± standard error (S.E). Those denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, ***$P \leq 0.001$

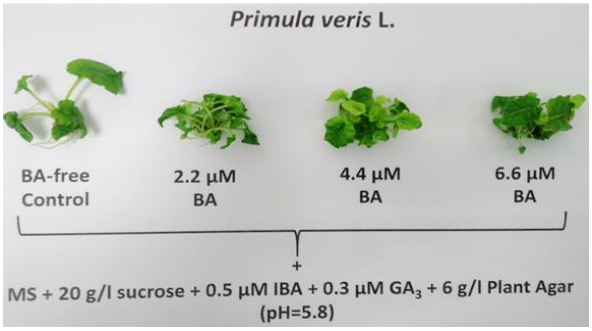

Fig 1: Effect of BA concentration (0, 2.2, 4.4, 6.6 µM) applied in combination with 0.1 µM IBA and 0.3 µM GA_3 on micro propagation of *Primula veris* subsp. *veris* shoot-tip explants after 3 weeks of culture in MS medium with 20 g/L sucrose and 6 g/L plant agar

The incorporation of IBA in the medium, regardless of its concentration (2.5 and 5 µM) gave optimum rooting results by exhibiting 100% rooting percentage with 5.78-6.88 roots/rooted microplant that were 0.71-0.78 cm long, without statistically significant difference. Root length was greater (1.94 cm) in the control treatment (IBA-free). In all three treatments, no multiple shoot production was observed. IBA increased the number of roots (x 2.63-3.14 times) and rooting percentage by 6.25% (from 93.75 to 100%) whereas it caused a substantial decrease in the length of roots by 1.17-1.23 cm (x 2.5-2.75 times), with respect to the control (93.75% rooting, 2.19 roots 1.94 cm long). In addition, IBA negatively affected the growth of the initial explant (0.72 cm of control to 0.53 of IBA treatments) (Table 3, Fig. 2).

Table 3: Effect of IBA concentration (0, 2.5 and 5 µM) on rooting (%), number of roots/ microplant, root length (cm), number of shoots/ explant and shoot length (cm) of *Primula veris* subsp. *veris* explants after 3 weeks of culture in MS medium with 20 g/L sucrose and 6 g/L plant agar

IBA (µM)	Rooting (%)	Root number / rooted microplant	Root length (cm)	Shoot number / explant	Shoot length (cm)
0	93.75 b	2.19 ± 0.29 b	1.94 ± 0.18 a	1.00 ± 0.00 a	0.72 ± 0.06 a
2.5	100 a	5.75 ± 0.74 a	0.77 ± 0.03 b	1.00 ± 0.00 a	0.53 ± 0.03 b
5	100 a	6.88 ± 0.82 a	0.71 ± 0.03 b	1.00 ± 0.00 a	0.53 ± 0.03 b
p-values	0.006**	0.000***	0.000***	1.000 ns	0.006**

Means (n=16) ± standard error (S.E.). Those denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$; ns: $P \geq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

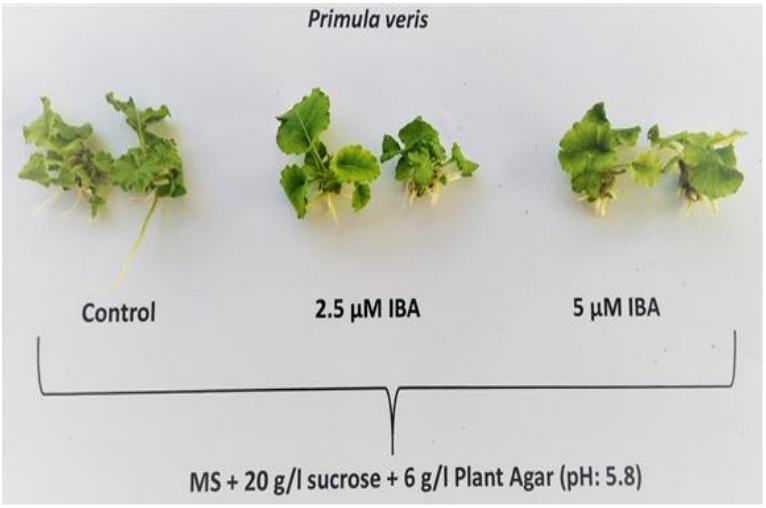

Fig 2: Effect of IBA concentration (0, 2.5 and 5 µM) on *in vitro* rooting of *Primula veris* subsp. *veris* after 3 weeks of culture in MS medium with 20 g/L sucrose and 6 g/L plant agar

3.3. Acclimatization of *in vitro* produced plantlets

All rooted microplants derived were successfully acclimatized irrespective of IBA concentration after four weeks of maintenance in the mist, therefore 100% survival rate was achieved (Fig. 3a-3g). During the summer season (mid-June 2019), the acclimatized plantlets bloomed, and the flowering period was extended for two months until mid-August 2019 (Fig. 3f-3g).

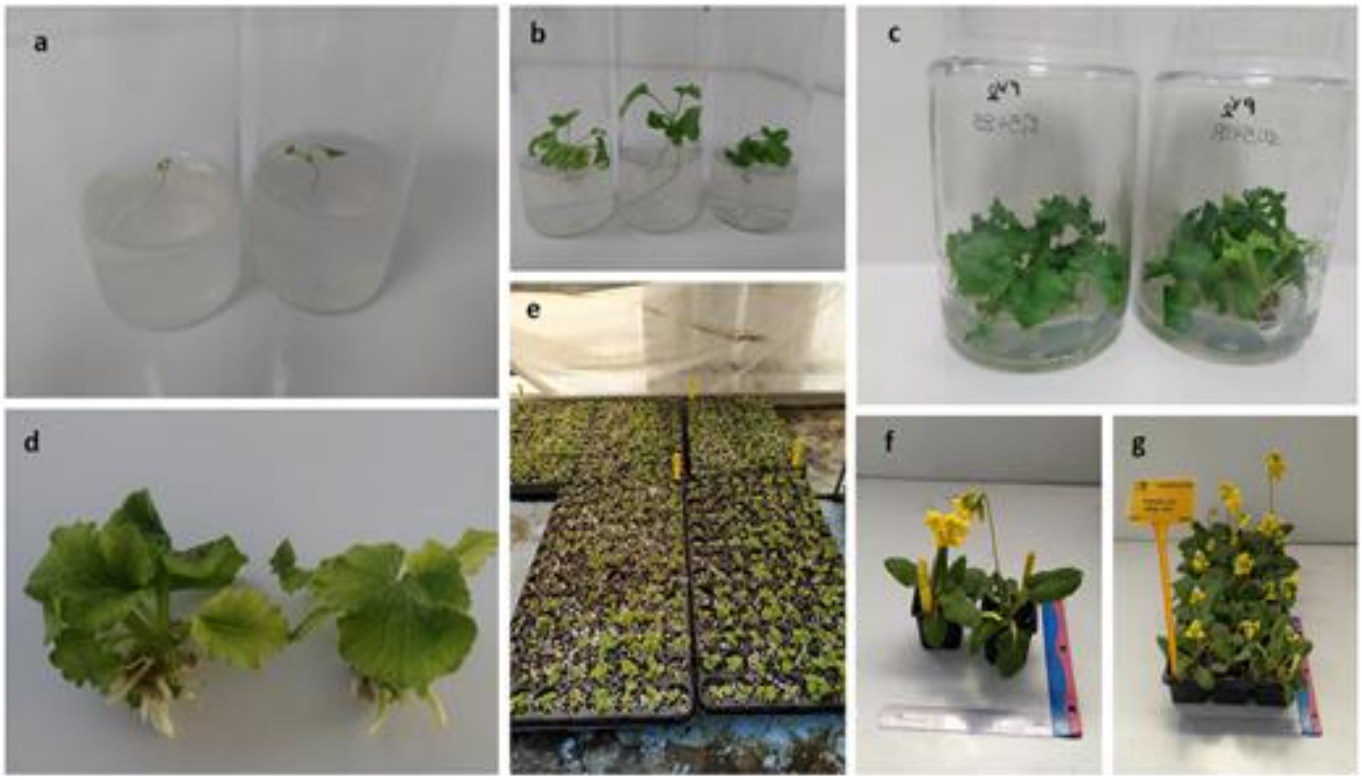

Fig 3: Micropropagation of *Primula veris* subsp. *veris*: (a) germination 30 days after initiation of culture; (b) germination 60 days after initiation of the culture; (c) proliferation stage; (d) rooted plantlets derived from treatment with 2.5 µM IBA; (e) acclimatized plants after 30 days; (f, g) acclimatized plants in blossom after 90 days

4. Discussion

4.1. *In vitro* seed germination and establishment of culture

Germination of seeds of *P. veris* and *P. heterochroma* has been reported to be difficult (Coumans *et al*., 1979; Draxler *et al*., 2002; Morozowska, 2002) [3-5], and sometimes endophytes occur in many cowslips and primroses which increase the risk of losing breeding material and seeds (Schween and Schwenkel, 2002) [16]. In the present study, *P. veris* subsp. *veris* seeds collected in 2001 and 2004 from wild-growing populations showed no germination potential, suffering bacterial and fungal infections. The absence of germination ability is likely due to the long maintenance without excluding poor seed quality originally. According to Brys *et al*. (2003) [19], seeds can be retained in seed banks and remain viable for more than three years. The wild cowslip seeds of 2010 tested in this study were stored for a longer period (8 years) and gave quite good results.

Gibberellins (including GA_3) are tetracyclic diterpenoid acids involved in various developmental and physiological processes in plants, such as seed activation, germination, growth of shoots, leaves and fruits, promoting flowering and other plant growth phases (Bottini *et al*., 2004) [20]. In this study, the use of GA_3 proved to be a major factor playing an important role in seed germination of *P. veris* subsp. *veris*. In particular, when GA_3 was added in the culture medium, germination rates approached 50% (33-46%), regardless if seeds were pretreated in GA_3 solution or not, and irrespectively of the photoperiod regime. In accordance with our results, the largest number of *P. veris* subsp. *veris* seedlings had been obtained on MS medium supplemented with GA_3 (2.90 µM) and KIN (2.33 µM) (Morozowska and WesŁowska, 2004) [12]. In the current study when there was no GA_3 in the substrate, germination rates were quite low (0-15%); however, in the case of GA_3-pretreated seeds, the germination rate was relatively higher approaching 46%. According to Morozowska (2002) [5], GA_3 (0.9 and 1.8 x 10^{-3} M) promotes dormancy break of *P. veris* seeds under laboratory conditions. Our results also showed that the percentage of germinated seeds was up to 46% when GA_3 was added in the medium, whereas the time required for germination of the maximum number of seeds varied from 25 to 60 days. In both photoperiod regimes (16 h light/8 dark, 24 h dark), the pretreatment of seeds in GA_3 solution for 20 min prior to their culture in plain MS medium, caused a four-fold increase in the final germination rate after 60 days of inoculation (from 6 to 26%) under 16 h light conditions, while in darkness resulted in 15% germination compared to the complete inhibition of germination with no pretreatment. Cerabolini *et al*. (2004) [21] reports that *P. glaucescens* Moretti seeds germinate in both sterile and non-sterile conditions and, although total germination is not affected by GA_3 concentration, doses of 10–500 mg/L promote equally earlier and more rapid germination.

Photoperiod (16 h light/8 dark, 24 h dark) significantly affected the germination capacity of *P. veris* subsp. *veris* seeds under experimentation. The pre-immersion of *P. veris* subsp. *veris* seeds in GA_3 solution for 20 min exhibited similar germination rates (46%) in 16 h photoperiod but led to a 3.5-fold decrease in total darkness compared to no

pre-treatment and culture in medium containing 250 mg/L GA_3. Our results are partly in agreement with those reported in *P. heterochroma* seeds showing no germination within 70 days on plant growth regulators free (PGRs-free) medium, whereas they germinate (17%) after 49 days on medium enriched with GA_3 (Hamidoghli *et al*., 2011) [14].

4.2. *In vitro* culture proliferation and rooting

In this study, BA irrespective of concentration had a stimulating effect on shoot formation (100%) of *P. veris* subsp. *veris* explants comparing to the control where no multiple shoot induction was achieved. Similar results have been reported by Borodulina *et al*. (2001) [9], indicating that the highest multiplication coefficient was exhibited on medium B5 (Gamborg *et al*., 1968) [22] enriched with BA (10 µM), NAA (0.5 µM) and GA_3 (5 µM). The optimum results for *P. veris* subsp. *veris* regarding mean shoot number (3.7) and mean shoot proliferation rate (2.2) were obtained when explants were treated with 4.4 µM and 6.6 µM, accordingly. In consistency with our findings, the highest shoot regeneration frequency of *P. heterochroma* (93% and 94%) and the largest shoot number/ leaf were achieved on MS media containing 8.8 µM BA + 10.74 µM NAA and 9.08 µM TDZ + 5.4 µM NAA (Hamidoghli *et al*., 2011), respectively [14]. Therefore, the MS basal medium supplemented with 4.4-6.6 µM BA, 0.5 µM IBA and 0.3 µM GA_3 was proved to be the most appropriate combination treatment promoting direct shoot formation of *P. veris* subsp. *veris* using shoot-tip explants. Accordingly, in *P. heterochroma*, when the basal medium is supplemented with 4.4 µM BA or 0.91 µM TDZ in combination with 2.15 µM NAA show the highest shoot regeneration, leaf number and length (Sharaf *et al*., 2011) [23]. In cowslip species (*P. veris)*, Morozowska and WesŁowska (2004) [12] report that MS medium containing 4.4 µM BA and 1.13 µM 2,4-D enhances effectively the *in vitro* propagation from shoot tips.

In several *Primula* species including *P. cuneifolia, P. malacoides* and *P. obconica*, root formation *in vitro* has been referred on various basal culture media free of PGRs (Shimada *et al*., 1997; Mizuhiro *et al*., 2001) [8, 10]. In this study, rooting potential of *P. veris* subsp. *veris* explants was optimum giving 100% rooting, increased root number (6.4) and root length (1.82 cm) in the BA-untreated control explants (MS basal medium). In accordance with our findings, Borodulina *et al*. (2001) [9] and Mizuhiro *et al*. (2001) [8] have reported that rooting of several *Primula* species was best promoted on ½ MS basal medium PGRs-free. A progressive decline in all rooting parameters occurred in *P. veris* subsp. *veris* explants under experimentation by increasing BA concentration which resulted in complete inhibition of root induction, thus rooting performance was best on the medium used as control, i.e. MS enriched with 0.5 µM IBA and 0.3 µM GA_3. In *P. heterochroma*, on the other hand, the addition of 4.4 µM BA or 0.91 µM TDZ plus 2.15 µM NAA in basal culture media positively influences rooting of microshoots (Sharaf *et al*., 2011) [23]. According to Hamidoghli *et al*. (2011) [14], the addition of either 0.88 µM BA + 10.74 µM NAA or 9.08 µM TDZ + 5.4 µM NAA in the culture medium used for this species may maximize the explants' root regeneration ability.

According to Coumans *et al*. (1979) [3], Borodulina *et al*. (2001) [9], and Schween and Schwenkel (2002) [16], the rooting performance of various *Primula* species has substantially been improved by using different auxin types (IBA and NAA). In this study, both IBA concentrations applied (2.5 and 5 µM) raised within three weeks the percentage of *P. veris* subsp. *veris* rooted microshoots to 100% and caused an increase (2.5-3-fold) in root number in comparison to the control. Similar rooting results have been obtained with the presence of 2.45 µM IBA to the MS medium (Morozowska and WesŁowska, 2004) [12]. On the other hand, the initiation of root primordia from adventitious microshoots of *P. vulgaris* is promoted after 10-15 days of culture in a different basal medium (WPM) when enriched with the same auxin type IBA at 2.7 µM (Hayta *et al*., 2016) [24]. In the current study of *P. veris* subsp. *veris*, the elongation of initiated roots was diminished regardless the concentration of IBA, leading to a 2.5-2.7 decrease in root length of microshoots. In agreement up to a degree with our results, IBA has been reported to stimulate both induction and growth of root primordia in several *Primula* spp. (Schween and Schwenkel, 2003; Hamidoghli *et al*., 2011; Jia *et al*., 2014) [14, 17, 25].

MS medium contains two nitrogen salts, NH_4NO_3 (20 mM) and KNO_3 (20 mM), leading to a nitrogen form ratio NH_4/NO_3 (20:40) exhibiting a 60 mM combined total, which is a reasonably high nitrogen concentration, especially for the majority of woody species (Vinterhalter *et al*., 2007) [26]. In all experiments conducted with *P. veris* subsp. *veris* shoot-tip explants in MS as the basal culture medium, no sign of callus formation was observed in any treatment. Similarly, in primrose (*P. vulgaris*) leaf explants, no callus formation is reported in MS basal medium despite the range of PGRs used, whereas callus induction is promoted in medium containing B5 macro salts with 966 mg/L KNO_3, half-strength MS micro salts, and MS vitamins with 30 g/l maltose (Hayta *et al*., 2016) [24].

4.3 Acclimatization of *in vitro* produced plantlets

In the present study with *P. veris* subsp. *veris*, all rooted plantlets that were transplanted to pots containing peat: perlite (1:1) mixtures were successfully acclimatized after four weeks under mist, exhibiting 100% survival rate. Our findings are in consistency with those presented for *P. heterochroma* where several rooted plantlets have been transplanted to pots containing a perlite, sand and soil (1:1:1) mixture and have been successfully acclimatized under high humidity conditions at room temperature (Hamidoghli *et al*., 2011) [14]. The rooted *P. veris* subsp. *veris* plantlets derived *in vitro* from the culture medium containing the highest IBA concentration (5 µM) had a better vegetative growth performance and root system development in terms of root thickness. In addition, the rooted plants that were obtained *in vitro* from the culture medium containing 2.5 µM IBA had more intense green leaf colour, greater plant stamina and vigour, greater leaf number and leaf size. Taking into consideration all macroscopic rooting attributes simultaneously and the cost of the culture media used, the addition of 2.5 µM IBA in the MS medium containing 20 g/L sucrose and 6 g/L plant agar was the most cost-effective treatment promoting the *in vitro* rooting of *P. veris* subsp. *veris* explants within three weeks. Similar observations to ours with respect to plant morphology have been found by Morozowska and WesŁowska (2004) [12]; in their study, the *P. veris* plantlets are reported with well-shaped rosettes with several leaves

and normally developed rootlets when the medium is supplemented with IBA, while when developed on medium without growth regulators, they are reported with thin and elongated rootlets. According to the same authors, *in vitro* culture of *P. veris* subsp. *veris* has lasted 8–10 weeks from the moment the seeds were placed on the medium, including seed germination, proliferation, and growth until shoot rooting (Morozowska and WesŁowska, 2004) [12]. In our study, the whole process from seed disinfection and initial establishment to rooting of *P. veris* subsp. *veris* shoot-tip explants was completed within 18 weeks (8 for seed germination, 3 for shoot proliferation, 3 for root formation and another 4 for *ex vitro* acclimatization and hardening).

5. Conclusions

GA_3 was the main factor that affected the germination capacity of the cowslip seeds. The germination capacity of *P. veris* subsp. *veris* seeds stored for more than 10 years was limited to zero, while those stored for a shorter period (8 years) germinated at approximately 50%. The growth and shoot development of the derived seedlings was faster and better when seedlings were grown under 16 h-photoperiod compared to 24 h darkness. In addition, the use of GA_3 enhanced the germination procedure of seeds stored for a long period. The long storage life (> 10 years) of *P. veris* subsp. *veris* seeds (4-5ºC, RH < 5%) adversely affects their germination, therefore their renewal in conservation seed banks should be made at shorter intervals. The establishment of a micropropagation protocol for *P. veris* subsp. *veris* is a key step in generating mass propagation material for systematic cultivation with a view to its sustainable exploitation; this may alleviate the over-collection pressure on declining wild-growing populations. The effective *in vitro* propagation can be achieved by seed germination on a MS medium with 250 mg/L GA_3, proliferation on MS medium with 4.4-6.6 µM BA, 0.5 µM IBA and 0.3 µM GA_3 (approximately 3.5 shoot proliferation rate/three weeks) and rooting on MS with 2.5-5 µM IBA (100%, three weeks) with 100% survival rate during acclimatization of rooted microplants under *ex vitro* conditions.

6. References

1. Dimopoulos P, Raus Th, Bergmeier E, Constantinidis Th, Iatrou G, Kokkini S, *et al*. Vascular plants of Greece: An Annotated Checklist. Englera. 2013; 31. Botanischer Garten und Botanisches Museum Berlin-Dahlem and Freie Universität Berlin and Hellenic Botanical Society, Athens, Greece.
2. Länger R. Assessment report on *Primula veris* L. and/or *Primula elatior* (L.) Hill, flos. Committee on Herbal Medicinal Products (HMPC), EMA/HMPC/136583/ 2012, London, UK, 2012.
3. Coumans M, Coumans-Gillcs MF, Delhez J, Gaspar T. Mass propagation of *Primula obconica*. Acta Horticulturae. 1979; 91:287-293. https://doi.org/10. 17660/ActaHortic.1979.91.33
4. Draxler L, Göhler I, Franz C. Cultivation and breeding of cowslip (*Primula veris*) for producing medicinal drugs. Revista de Fitoterapia. 2002; 2(1):327.
5. Morozowska M. Some factors affecting viability, dormancy and germination of *Primula veris* (L.) seeds. Folia Horticulturae. 2002; 14(1):243-253. https:// eurekamag.com/research/003/938/003938820.php
6. Bajaj YSP. Regeneration of plants from ultra-low frozen anthers of *Primula obconica*. Scientia Horticulturae. 1981; 14:93-95. https://doi.org/10.1016/ 0304-4238 (81)90081-9
7. Merkle S, Götz EM. Micropropagation of inbred lines of *Primula acaulis* for breeding purposes. Abstracts of 7th Congress Plant Tissue Cell Cultures, Amsterdam, The Netherlands. 1990; 116:24-29.
8. Mizuhiro M, Kenichi Y, Ito K, Kadowaki S, Ohashi H, Mii M. Plant regeneration from cell suspension-derived protoplasts of *Primula malacoides* and *Primula obconica*. Plant Science. 2001; 160:1221-1228. https://doi.org/10.1016/S0168-9452(01)00390-9
9. Borodulina ID, Vechernina NA, Dolganova ZV. *In vitro* propagation of two hybrid *Primula* L. species. Rastitel'nye Resursy. 2001; 4:114-122 (in Russian).
10. Shimada T, Matsushita T, Otani M. Plant regeneration from leaf explants of *Primula cuneifolia* var. *hakusanensis*, "Hakusan-kozakura". Plant Biotechnology. 1997; 14:47-50. https://doi.org/10.5511/ plantbiotechnology.14.47
11. Benson EE, Danaher JE, Pimbley IM, Anderson CT, Wake JE, Daley S, *et al*. *In vitro* micropropagation of *Primula scotica*: A rare Scottish plant. Biodiversity and Conservation. 2000; 9:711-726. https://doi.org/10.1023/ A:1008941726419
12. Morozowska M, Wesołowska M. *In vitro* clonal propagation of *Primula veris* L. and preliminary phytochemical analysis. Acta Biologica Cracoviensia Series Botanica. 2004; 46:169-175
13. Takihira M, Otani M, Tsuchiya S, Shimada T. Plant regeneration from leaf explants of auricula cultivars (*Primula pubescens* Jacq.). Plant Biotechnology. 2007; 24:425-427.https://doi.org/10.5511/plantbiotechnology. 24.425
14. Hamidoghli Y, Sharaf ARN, Zakizadeh H. Organogenesis from seedling derived leaf explants of primrose (*Primula heterochroma* Stapf.) as a scarce Iranian plant. Australian Journal of Crop Science. 2011; 5:391-395.
15. Yamamoto T, Magaya Y, Maruyama Y. Mass propagation of *Primula sieborldii* E. Morr. through leaf segment culture. The Bulletin of the Faculty of Horticulture, Minami Kyusyu University. 1999; 29(A):9-14.
16. Schween G, Schwenkel HG. *In vitro* regeneration in *Primula* ssp. via organogenesis. Plant Cell Reports. 2002; 20:1006-1010. https://doi.org/10.1007/s00299-00 2-0443-2
17. Schween G, Schwenkel HG. Effect of genotype on callus induction, shoot regeneration, and phenotypic stability of regenerated plants in the greenhouse of *Primula* ssp. Plant Cell, Tissue and Organ Culture. 2003; 72:53-61. https://doi.org/10.1023/A:102122741 4880
18. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tissue culture. Physiologia Plantarum. 1962; 15:473-497. https://doi.org/10.1111/ j.1399-3054.1962.tb08052.x
19. Brys R, Jacquemyn H, Endels P, Hermy M, DE Blust G. The relationship between reproductive success and demographic structure in remnant populations of *Primula veris*. Acta Oecologica. 2003; 24:247-253. https://doi.org/10.1016/j.actao.2003.09.002

20. Bottini R, Cassán F, Piccoli P. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. Applied Microbiology and Biotechnology. 2004; 65:497-503. https://doi.org/10.1007/s00253-004-1696-1
21. Cerabolini B, De Andreis R, Ceriani RM, Pierce S, Raimondi B. Seed germination and conservation of endangered species from the Italian Alps: *Physoplexis comosa* and *Primula glaucescens*. Biological Conservation. 2004; 117:351-356. https://doi.org/10.1016/j.biocon.2003.12.011
22. Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspensions cultures of soybean root cells. Experimental Cell Research. 1968; 50:151-158. https://doi.org/10.1016/0014-4827(68)90403-5
23. Sharaf ARN, Hamidoghli Y, Zakizadeh H. *In vitro* seed germination and micropropagation of primrose (*Primula heterochroma* Stapf.) an endemic endangered Iranian species via shoot tip explants. Horticulture, Environment and Biotechnology. 2011; 52(3):298-302. https://doi.org/10.1007/s13580-011-0129-1
24. Hayta S, Smedley Ma, Li j, harwood WA, Gilmartin p. Plant regeneration from leaf-derived callus cultures of primrose (*Primula vulgaris*). HortScience. 2016; 51(5):558-562. https://doi.org/10.21273/HORTSCI.51.5.5 58
25. Jia Y, Zhang QX, Pan HT, Wang SQ, Liu GL, Sun LX. Callus induction and haploid plant regeneration from baby primrose (*Primula forbesii* Franch.) anther culture. Scientia Horticulturae. 2014; 176:273-281. https://doi.org/10.1016/j.scienta. 2014.07.018
26. Vinterhalter B, Ninković S, Zdravković-Korać S, Subotić A, Vinterhalter D. Effect of nitrogen salts on the growth of *Ceratonia siliqua* L. shoot cultures. Archives of Biological Science Belgrade. 2007; 59(3):217-222. https://doi.org/10.2298/ABS0703217V