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Research Article

In vitro germination and regeneration of *Senna artemisioides*, a valuable leguminous ornamental shrub

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Abstract

This research was carried out at the prospect of introducing new ornamental species in the floriculture industry concerning the use of valuable plants. A leguminous species native to Australia was the object of the present study. Seeds of *Senna artemisioides* established in a private botanical garden were chosen as the starting material for both germination and *in vitro* studies. Hot water treatment at 90 °C, for 10 min was applied to interrupt the seed-coat dormancy. Incubation was conducted *in vitro* in Petri dishes, at seven different temperatures ranging from of 5.0 to 35.0 °C, involving three separate treatments of incubation at 5.0 °C, for 0, 30 and 60 days. The seeds germinated at 25 °C (56.0%). Node explants derived from produced seedlings were cultured *in vitro* onto Murashige and Skoog media (MS), hormone free (Hf) or containing 6-Benzyladenine (BA). The establishment of initial cultures was successful being higher on MS supplemented with BA at the range 0.1-1.0 mg L^{-1} media. The multiplication phase followed on MS media containing the same range of a total of four cytokinins widely used in *in vitro* experimentation, i.e BA, Kinetin (KIN), N6-Isopentenyladenine (2IP) and Zeatin (ZEA). 1-Naphthaleneacetic acid (NAA) was tested in conjunction with the higher concentration of the four cytokinins. MS media supplemented with BA at 0.5 mg L^{-1} led to a satisfactory proliferation rate; 2.4 shoots/explant were formed. Rooting phase had moderate efficiency, being higher for media with 4.0 mg L^{-1} IBA. Acclimatisation was successful for all rooted plantlets, promising an effective method for exploitation of this new ornamental species of high value.

Keywords: acclimatisation; *in vitro* rooting; micropropagation; new ornamental; tissue culture

Introduction

The genus *Senna* (syn. *Cassia* L.) is one of the largest genera in the Fabaceae family (legume family) with 351 spp (Randell and Barlow, 1998; IPNI 2022). It is widely distributed throughout Australia with endemic species; it can be found sparsely in other countries as well (Randell, 1989; Holman and Playford, 2000; Marazzi and Sanderson, 2010). *Senna artemisioides* (Gaudich. ex DC.) Randell is an evergreen shrub up to 2.0 m height (Figure 1A). The species name was first published in 1989 (Randell, 1989). The plant is very important

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providing habitat for a number of arthropods in shrubland ecosystems of Australian woodland (Kwok and Eldridge, 2016). It has grey-green, rather silver, leaves divided into linear leaflets, and it is widely known as silver senna, wormwood or feathery senna. It is impressive during flowering forming countless, stunning, yellow flowers (Luckow 1996; RHS Plant Selector, 2021), from early spring until early summer (Figure 1B). During the summer it has also very attractive, numerus fruits, brown in colour which make it visually interesting (Figure 1C). The plant is of high ornamental value, grows itself in a wide range of habitats of mainland Australia, from rocky to sandy areas and shows drought resistance due to its adaptation to the arid Australian climate. In the context of the utilization of new species from productive floriculture and the needs of the market that seeks new species suitable for the new conditions shaped by climate change, the interest is focused on species from arid environments as is the case with plants of the Mediterranean flora. In the last decades a series of studies and the suitable strategies on introduction of new species as ornamental plants have been published (Tsoktouridis *et al*., 2019; Bertsouklis and Panagaki, 2022; Papafotiou *et al*., 2022; Pipinis *et al*., 2022).

Figure 1. *Senna artemisioides* mother plant, 7 years old, cultivated in a private botanical garden, at Peania (37°57'53.0"N 23°51'10.0"E, Attica, Greece), in September 2020 (A) blooming in May (B) fruiting in August (C)

A simple, cost effective and quick protocol is crucial for achieving practical goals of exploitation of new species. The *in vitro* seedling method significantly reduces time taken to initiate *in vitro* cultures bypassing the explant preparation step (Ahmad *et al*., 2021). Starting from seeds is a widespread method to establish *in vitro* cultures of new ornamental species of high proliferation rate (Papafotiou *et al*., 2013; Cabahug *et al*., 2018). Seed propagation also enhances the genetic diversity within species, which subsequently contributes to the selection of genotypes of high ornamental and commercial value (Sarasan *et al*., 2011; Generoso *et al*., 2019; Silva *et al*., 2021). Efficient clonal propagation strategies are also necessary in order to meet the increasing demands of the ornamental plant market. Molecular methods could be employed, for the assessment of genetic diversity and the relationship between individuals aiming at the preservation of natural variation.

More than 85% of plant species growing in arid environments produce dormant seeds (Baskin and Baskin, 2003), thus understanding of seed dormancy is of high importance for restoration projects (Allan *et al*., 2004). Fabaceae species usually have hard seeds or physically dormant seeds (Baskin *et al*., 2000). Physical dormancy of seeds is due to their water-impermeable seed coat. A single layer of lignified Malpighian cells in

the outer integument prevents the imbibition of the water (Rolston, 1978). Entry of water through the seed coat in legumes is usually regulated by an anatomical structure referred to as a water gap within the impermeable layer, which "opens" under suitable environmental conditions, allowing imbibition (Baskin and Baskin, 2003).

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and woody plant medium (WPM; McCown and Lloyd, 1981) have been used in previous studies on micropropagation of other *Senna* species (Fett-Neto *et al*., 2000; Agrawal and Sardar, 2006; Gbadamosi and Hassan, 2013; Parveen and Shahzad, 2014; Siddique *et al*., 2015; Ahmed *et al*., 2013). There are no studies on their effectiveness for micropropagation of *S. artemisioides*. The cytokinin type of the culture medium induced significantly different responses in other *Senna* species, during both the culture establishment and shoot multiplication phase (Gbadamosi and Hassan, 2013). Cases of dormancy-breaking and *ex vitro* germination of other *Senna* species have indeed been reported (Baskin *et al*., 1998; Pound *et al*., 2014). Up to now neither seed germination nor shoot multiplication directly from *in vitro* seedlings of *S. artemisioides* have ever been reported. The aim of the present study was to develop an efficient protocol for *in vitro* propagation of *S. artemisioides* starting from *in vitro* grown seedlings. In addition, the *in vitro* germination of this plant was studied so that it could be introduced for use in European Floriculture industry and Landscape Architecture.

Materials and Methods

Plant material

Seeds were collected fully mature, from an adult, well grown, mother plant, 7 years old, 1.80 m in height, cultivated in a private botanical garden, at Peania (37°57'53.0"N 23°51'10.0"E, Attica, Greece), in September 2020 (Figure 1). The seeds were inside the fruits (pods) which had been dried and opened for seed dispersal, indicating their complete ripening (Baskin and Baskin, 1998). Then the seeds were transferred to the Laboratory of Floriculture and Landscape Architecture, Agricultural University of Athens, Athens. The seeds were stored for 3 months dry, in the dark, in paper bags, at T=25.0 °C at a relative humidity of 30.0%. The seed pericarp was separated manually before the use of seeds in experimental treatments. A total of 4200 seeds were used for the germination studies (20 seeds/Petri dish, 100 seeds per each combination of temperature and treatment). For *in-vitro* micropropagation *in-vitro* grown seedlings were used as starting planting material.

In vitro germination

The seeds were placed for germination *in vitro*, in 9-cm plastic Petri dishes, with a solid medium of half strength Murashige and Skoog (1962) salt substrate (MS/2). They incubated in chambers of stable conditions, at seven different temperatures (5.0, 10.0, 15.0, 20.0, 25.0, 30.0 or 35.0 °C) and 16.0 h photoperiod under 37.5 μmol m^{-2} s^{-1} fluorescent light. Because treatments with high temperature are used to break dormancy of many Fabaceae (Auld and O'Connell, 1991; Pound *et al*., 2014), we immersed the seeds in warm water of an initial temperature of 90.0 °C; then seeds were allowed to cool for 12 h, for the scarification of the seed coat and the breaking of the seeds coat dormancy. The seeds first were surface sterilized with absolute alcohol solution (90.0%, v/v) for 10 sec and then with 25.0% solution (v/v) of commercial bleach (4.6% w/v sodium hypochlorite for 10 min. Afterwards they were rinsed three times, 3.0 min each, with sterile, distilled water. Three different treatments (T) were performed, and the seeds were placed for *in vitro* germination either directly (T1) or after incubation at low temperature (5.0 °C) after 30 or 60 days (T2 and T3 respectively). Seeds without hot water treatment (C1, C2, C3) and treated seeds (T1, T2, T3) were cultured in Petri dishes after 0, 30 and 60 days respectively. In compliance with the rules of the International Seed Testing Association (1999), germination was defined as the appearance of a radicle that would be at least 2.0 mm long and would be recorded every 2 d. T_{50} was defined as time taken by cumulative germination to reach 50.0% of its maximum

(Soltani *et al.*, 2001). One hundred seeds were used per treatment (five Petri dishes per treatment/20 seeds per Petri dish). The germination speed index (GSI) was calculated using the formula proposed by Maguire (1962):

GSI = G1/N1 + G2/N2 + ... + Gn/Nn

in which: G1, G2 and Gn = number of normal seedlings, computed during the first, second and last counts; and N1, N2, Nn = number of sowing days during the first, second and last counts.

All seeds that had failed to emerge were evaluated for viability using a cut-test on the 30th day; Seeds with a plump, firm, and white-greenish embryo were considered as viable (Carrugio, 2020).

Establishment of initial cultures and multiplication phase

S. artemisioides explants (shoot tips and single node explants, 0.6 cm long) derived from *in vitro* grown seedlings on MS/2 media, were cultured on hormone free MS media (Hf) or supplemented with BA at 0.1, 0.5 and 1.0 mg L^{-1} (initial culture).

Three subcultures followed and single node explants were cultured on MS Hf or supplemented with various cytokinins types, i.e., zeatin (ZEA), 6-benzyladenine (BA), kinetin (KIN), and 6-γ-γ-(dimethylallylamino)-purine (2IP) and concentrations. i.e., 0.1, 0.5 and 1.0 mg L^{-1} without or with naphthaleneacetic acid (NAA) at 0.05 mg L^{-1}. The concentrations of plant growth regulators used were based on the results of previous studies on other *Senna* species (Parveen and Shahzad, 2014; Siddique *et al.,* 2015).

In vitro rooting

Micro-shoots, 1.5 cm long, were produced by sub-culturing on various media during the multiplication phase, were transferred for rooting onto half-strength MS (MS/2), containing 0.0, 0.5, 1.0, 2.0 and 4.0 mg L^{-1} indole-3-butyric acid (IBA).

In vitro culture conditions and data collection

In vitro cultures of the initial and multiplication phases took place in 380.0 mL glass vessels (six explants per vessel). The cultures were maintained at 25.0 °C, with a 16 h photoperiod at 37.5 μmol m^{-2} s^{-1} provided by cool-white, fluorescent lamps. The media were solidified with 8.0 g L^{-1} agar and the pH of all media was adjusted to 5.7–5.8 before addition of the agar and autoclaving (121.0 °C for 20 min). Data on the cultures were collected after 40 days of culture regarding shoot proliferation percentages, shoot numbers per explant, shoot lengths and node number per shoot. The "multiplication index" (MI) was calculated by multiplying the percentage of explants which produced shoots by the mean number of shoots per responding explant, then divided by 0.6 (the length of each explant used for sub-culture) to obtain the proliferation potential of the cultures.

Ex vitro acclimatisation

Rooted micro-shoots 1.5 cm long were rinsed under running tap water. Then they were transferred to containers (500 mL, eight plantlets per container), on peat (pH 5.5-6.5, Klasmann-Delimann GmbH, Geeste, Germany) and perlite (particles diameter 0.1-0.5 cm, Perloflor, Isocon S.A., Athens, Greece) substrate 1:1 (v/v). Each container contained 8 micro-shoots (a total of 24 plantlets were planted for acclimatisation). The containers were covered with transparent plastic wrap (Sanitas) to maintain humidity. Then they were placed for one week in a growth chamber (25.0 °C, 16-h cool white fluorescent light 37.5 μmol m^{-2} s^{-1}/8-h dark photoperiod) and were uncovered one week later. They remained one more week and then they were transferred to a heated glasshouse (37°58'58.0" N, 23°42'19.2" E) and placed on a greenhouse bench for one more week. At the end of that period, data on acclimatisation were recorded. The plants were transplanted singly in 1.1 L plastic pots with peat: perlite (1/1, v/v) and fertilized monthly with 2.0 g L^{-1} complete water-soluble fertilizer (Nutrileaf 60, 20-20-20; Miller Chemical and Fertilizer Corp., Hanover, PA, USA). The final survival rate was recorded two months later.

Experimental design and statistical analysis

The statistical analysis used the completely randomized design method, and the significance of the results was tested by one-way analysis of variance (ANOVA). Data on percentages were arcsine-transformed prior to statistical analysis. The treatment means were compared via use of the Tukey test at $p < 0.05$ (JMP 14.0 software, SAS Institute Inc., Cary, NC, USA, 2013). As shown on the data tables, the number of replicates per treatment differed between experiments. Experiments on the multiplication phase involved three subcultures and data for the purposes of our statistical analysis were pooled. Principal coordinate analysis (PCA) was used to assist in visualizing the data. Germination rates were expressed as (mean ± standard error).

Results

In vitro germination

The disinfection method of seeds resulted in a 95.0% success rate. Non-scarified seeds (C1, C2, C3) germinated at the range between 2.0% - 6.4% for the range of 15 °C-35 °C with no difference, after incubation at 0 days, 30 days or 60 days at low temperature (5.0 °C). Regarding the germination percentages for scarified seeds, the highest germination percentage was 56.0% at 25 °C for the T1 treatment. The germination percentage proved similar, 47.0% and 41.4% for the T2 treatment at 20.0 °C and 25.0 °C respectively, as well, while for the T3 treatment the germination percentage was slightly higher, 34.3%, at 25.0 °C (Table 1; Figure 2). The germination percentage at the lower cardinal germination temperatures 10.0 °C was 5.0%, 2.9% and 3.5% respectively, being the lower one for each treatment. The germination percentage for the higher temperature tested (35 °C) was higher compared to the lower threshold for the T1 and T2 treatments (33.0% and 30.0% respectively); for the T3 treatment it was similar (6.4%) as that one of the lower threshold (Table 1; Figure 2).

T_{50} was completed in 3 and 4 days at 20.0 °C and 25.0 °C for the T1 treatment; T_{50} was completed in 4 and 2 days at 20.0 °C and 25.0 °C for the T2 treatment while it was increased to 5 days at 20.0 °C and 25.0 °C as well, for the T3 treatment (Figure 3). The germination speed index stood at 48.2, 52.5 and 32.1, at 25.0 °C for the T1, T2 and T3 treatments respectively (Figure 3). Germination was completed within 32.0 days. Non-germinated seeds, had a plump, firm, and white-greenish embryo and all of them were viable, in all the treatments.

Table 1. *In vitro* germination of *Senna artemisioides* at temperatures shown, after 0 (T1), 30 (T2) or 60 (T3) days of incubation. All seeds had been previously soaked in hot water; control seeds (C1, C2, C3) had not been treated with hot water

Temperature (°C)	Germination (%) ± SD					
	0 days		30 days		60 days	
	C1	T1	C2	T2	C3	T3
5	-	-	-	-	-	-
10	-	5.0 ± 2.8 d	-	2.9 ± 1.5 c	-	3.5 ± 2.1 c
15	2.4 ± 1.0 a	39.0 ± 6.0 bc	2.4 ± 1.0 a	36.5 ± 6.5 ab	2.0 ± 1.0 a	30.7 ± 3.2 ab
20	2.4 ± 0.8 a	45.0 ± 3.2 b	2.4 ± 0.8 a	47.0± 4.1 a	2.8 ± 0.8 a	32.9 ± 4.9 ab
25	4.0 ± 0.6 a	56.0 ± 3.7 a	4.0 ± 0.6 a	41.4 ± 4.6 a	4.0 ± 0.6 a	34.3 ± 4.1 a
30	6.4 ± 2.7 a	37.0 ± 4.4 bc	6.4 ± 2.7 a	37.0 ± 2.4 ab	6.4 ± 2.7 a	24.3 ± 2.3 b
35	4.0 ± 1.0 a	33.0 ± 4.4 c	4.0 ± 1.0 a	30.0 ± 4.9 b	2.4 ± 1.0 a	6.4 ± 2.1 c

Mean (± SE) separation in columns by Tukey test at $p < 0.05$, n=5, 20 seeds/Petri dish (total 100 seeds per treatment). Different letters denote significant differences (Tukey test, $p < 0.05$)

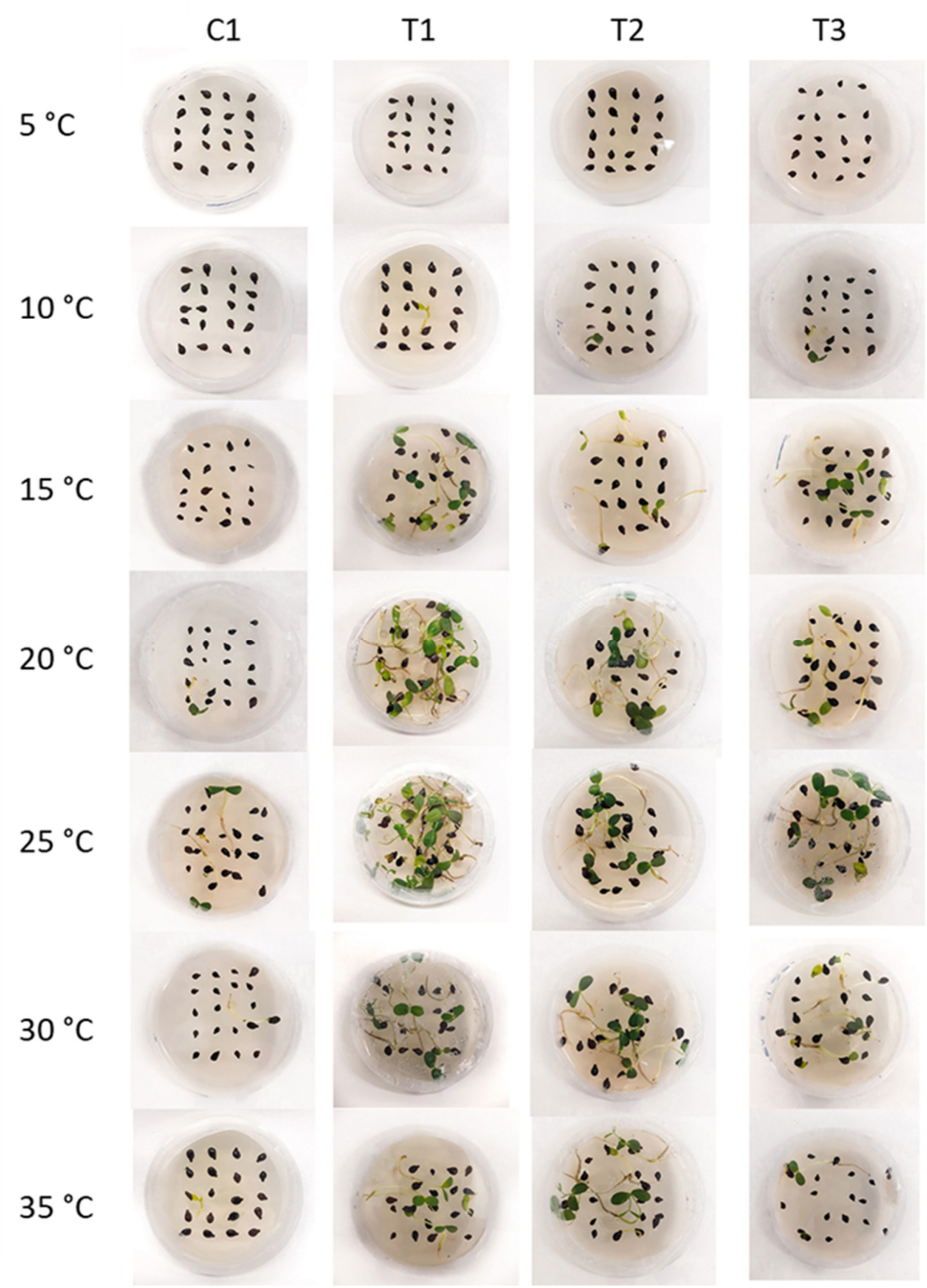

Figure 2. Non-scarified (C1) and treated seeds of *Senna artemisioides*, 3-months old, in Petri dishes, containing half strength MS, at 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 °C, under 16-h light/8-h darkness: after 0 (T1), 30 (T2) and 60 (T3) days of incubation at 5 °C

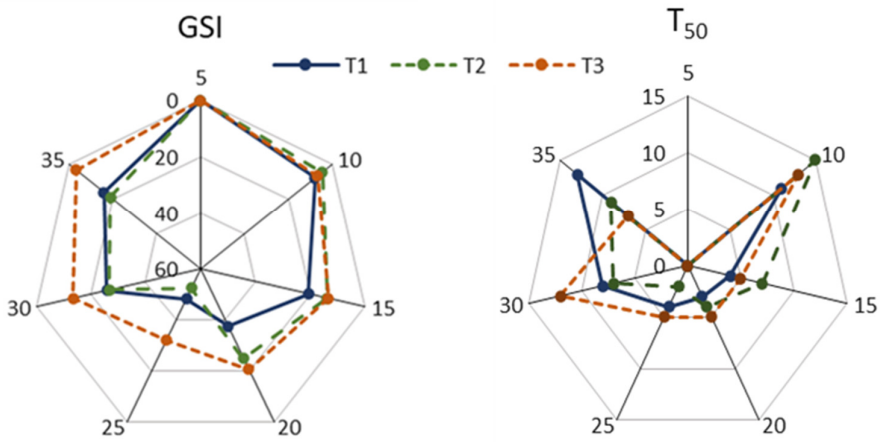

Figure 3. Germination speed index (GSI) and time to reach 50.0% of final germination (T_{50}) scores of *Senna artemisioides* seeds, treated with hot water, at seven different temperatures (5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 °C)
T1, T2, T3: 0, 30 and 60 days of incubation respectively, in the dark, at 5 °C

Establishment of initial cultures and multiplication phase

MS media proved effective for establishment of the initial cultures of single node *S. artemisioides* explants, with no differences in terms of shooting percentage (78.0%-97.0%) between different treatments (Table 2). Shoot number was higher (2.0-2.2 shoots/explant) for media with BA at 0.5 and 1.0 BA mg L^{-1}, while shoot length was higher (0.9 cm-1.2 cm) for media containing BA (Table 2), and MI as well. There was no difference between node number. The shooting percentage was high and ranged from 83.0% to 100.0% for all the cytokinins tested during the multiplication phase (Table 3). The shoot proliferation established when nodal explants, excised from micro-shoots produced on various substrates were sub-cultured on media containing BA at 1.0 mg L^{-1} or 0.5 mg L^{-1} without or with NAA at 0.05 mg L^{-1} NAA (2.4-2.5 shoots/explant were formed, Table 3). The higher shoot length (1.5 cm) was calculated for media containing BA at 0.1 mg L^{-1} and node number as well (5.4 nodes/explant). The MI was higher for media supplemented with 0.5 mg L^{-1} (Table 3, Figure 4A, B).

PCA analysis (Figure 5) transformed the original data of subcultures and different parameters in a set of uncorrelated new variables (principal components including eigenvalues>1). PCA produced two components, in declining order of importance and explained 80.04% of the total variability among the data of shoot-formation during multiplication stage. The first PC component (PC1) accounted for 57.4% of the total variation and was defined by shoot length and MI. The second PCA component (PC2) explained another 22.6% of the total variation and was defined by the shoot number, node number and shooting percentage (%) (Table 4), confirming the one-way analysis.

Table 2. Establishment of *Senna artemisioides* initial culture on MS media without hormone (Hf) or supplemented with BA at 0.1, 0.5 and 1.0 mg L^{-1} on shoot proliferation from shoot explants excised from *in vitro* young seedlings cultured on MS/2 media

BA (mg L^{-1})	Shooting (%)	Shoot number	Shoot length (cm)	Node number	MI
-(Hf) †	78.0 a	1.1 b	0.6 b	3.2 a	0.8 b
0.1	94.0 a	1.7 b	1.1 a	4.0 a	2.9 a
0.5	97.0 a	2.0 a	1.0 a	4.1 a	3.2 a
1.0	91.0 a	2.2 a	0.9 a	3.9 a	3.2 a
$F_{\text{one-way}}$	ns	***	**	***	***

Values followed by different lowercase letter within each trait are significantly different at the 5.0% level, determined by the one-way Anova (Tukey test, $p < 0.05$). n= 36; †Hf: hormone-free; ns: non-significant

Table 3. Effect of cytokinin type (BA, KIN, 2IP, ZEA) at three different concentrations (0.1, 0.5 and 1.0 mg L^{-1}) and the combined effect of BA/ NAA, KIN/ NAA, 2IP/ NAA, ZEA/ NAA at 0.5/ 0.05 mg L^{-1} on shoot production from node explants of *Senna artemisioides* on MS media

Cytokinin (mg L^{-1})	NAA (mg L^{-1})	Shooting (%)	Shoot number	Shoot length (cm)	Node number	MI
-(Hf) †	-	90.0 a	1.1 b	0.2 e	1.9 f	0.5 d
BA 0.1		90.0 a	1.6 b	1.5 a	5.4 a	3.6 ab
BA 0.5		97.0 a	2.4 a	1.1 b	4.8 ab	4.3 a
BA 1.0		91.0 a	2.5 a	0.8 c	3.7 c	3.0 b
BA 0.5	0.05	98.0 a	2.4 a	0.5 de	5.1 ab	2.0 c
KIN 0.1		88.0 a	1.2 b	0.3 de	2.4 ef	0.9 d
KIN 0.5		95.0 a	1.3 b	0.5 d	2.7 cde	1.0 d
KIN 1.0		92.0 a	1.1 b	0.5 d	2.8 cde	0.5 d
KIN 0.5	0.05	85.0 a	1.1 b	0.3 de	2.8 cde	0.6 d
2IP 0.1		92.0 a	1.2 b	0.3 de	2.4 ef	1.1 d
2IP 0.5		88.0 a	1.2 b	0.6 cde	2.9 cde	1.1 cd
2IP 1.0		88.0 a	1.1 b	0.7 cd	3.1 cde	1.1 cd
2IP 0.5	0.05	83.0 a	1.1 b	0.6 cde	3.6 cde	0.8 d
ZEA 0.1		100.0 a	1.2 b	0.4 de	3.1 cde	0.9 d
ZEA 0.5		96.0 a	1.1 b	0.5 d	2.9 cde	0.9 d
ZEA 1.0		96.0 a	1.5 b	0.5 cd	2.9 cde	1.2 d
ZEA 0.5	0.05	96.0 a	1.5 b	0.5 cd	3.4 cde	1.2 d
$F_{\text{one-way}}$		ns	***	***	***	***

Values followed by different lowercase letter within each trait are significantly different at the 5% level, determined by the one-way Anova (Tuckey test, $p < 0.05$). †Hf: hormone-free; n= 60-80; ns: non-significant

Table 4. Results of principal components calculation

Principal Components	
1	2
% Contribution of variability	
57.43	22.61
Related parameters	
Shoot length	Shoot number
MI†	Node number
	Shooting

†MI: Multiplication Index

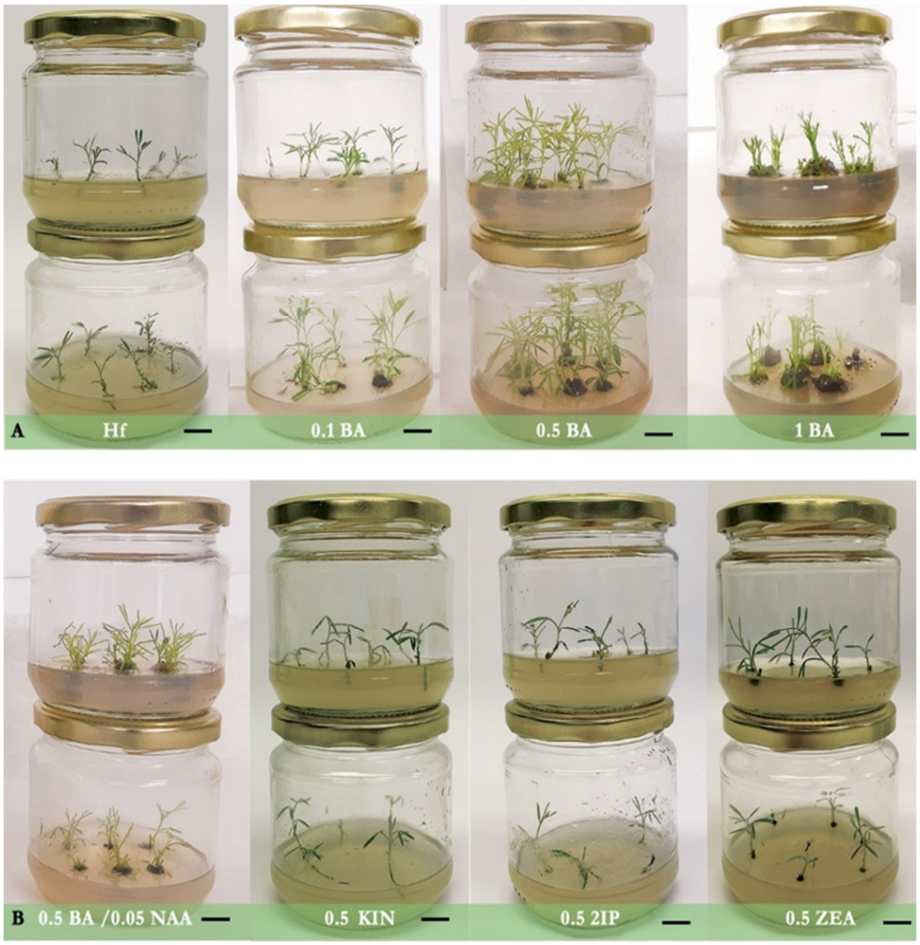

Figure 4. *In vitro* response of *Senna artemisioides* explants during multiplication phase, after 4 weeks of culture on MS medium without hormone (Hf), or supplemented with BA at 0.1, 0.5 or 1.0 mg L^{-1} (A) and 0.5/0.05 BA/NAA mg L^{-1} or KIN, 2IP or ZEA at 0.5 mg L^{-1} at 0.5 mg L^{-1} (B)
Bar represents a length of 1.0 cm

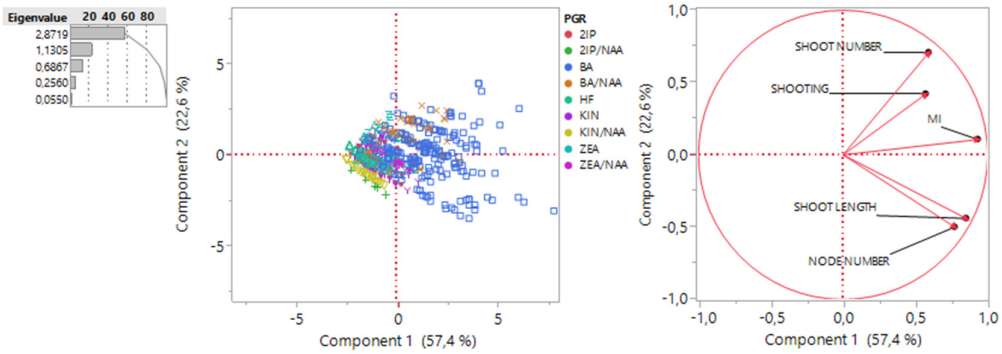

Figure 5. PCA of variables related to *in vitro* morphogenesis of *Senna artemisioides* in response to different media, without hormone (Hf) or supplemented with BA, KIN, ZEA or 2IP (at 0.1, 0.5 and 1.0 mg L^{-1}), without NAA or at 0.5 mg L^{-1} of each with NAA at 0.05 mg L^{-1}

In vitro rooting and ex vitro acclimatisation

Rooting of the micro-shoots derived from the multiplication phase took place at low percentages; the rooting percentage was higher (29.0%) on MS/2 media which were supplemented with 4.0 mg L^{-1} IBA (Table 5, Figure 6A). A decrease of rooting percentage was observed lowering the concentration of IBA, and a few roots were produced, higher at 0.5 and 1.0 mg L^{-1} IBA with no difference (3.0 and 2.0 roots respectively). Root length was higher on media containing IBA at 4.0 mg L^{-1}. Rooted micro-shoots were successfully (100.0%) acclimatised *ex vitro* (Figure 6B). After acclimatisation the plants were transplanted in pots and their survival rate after the transfer was again 100.0% (Figure 6C, D).

Table 5. *In vitro* rooting of *Senna artemisioides* micro-shoots on half-strength MS derived in rooting stage as affected by IBA concentration (mg L^{-1})

IBA (mg L^{-1})	Rooting (%)	Root number	Root length (cm)
-(Hf)†	-	-	-
0.5	7.0 b	3.0 a	3.0 a
1.0	10.0 b	2.0 a	2.2 b
2.0	21.0 ab	1.0 b	2.1 b
4.0	29.0 a	1.1 b	2.8 a
$F_{\text{one-way ANOVA}}$	*	*	*

Different letters in the same column indicate significant differences at the 5.0% level, determined by the one-way Anova (Tuckey test, $p < 0.05$); *, significant at $p < 0.05$, respectively, n= 25-35; †Hf=hormone-free

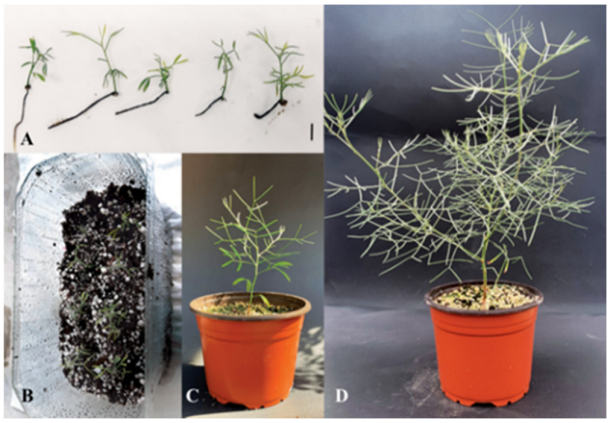

Figure 6. (A) *In vitro* rooted *Senna artemisioides* micro-shoots on media containing 2.0 mg L^{-1} IBA; (B) Plantlets during *ex vitro* acclimatisation on 1 peat:1 perlite (v/v). Two-month-old plant (C); Six-month old plant (D)
Bar represents a length of 1 cm

Discussion

The main aim of the present study focused on the regeneration of the *S. artemisioides* a valuable Leguminous ornamental plant, starting from young, *in vitro* grown seedlings. Meanwhile, the research focused on the assessment of the species' germinability using an easy and quick method; this method could be easily established to the floricultural industry considering that *S. artemisioides,* like many members of Fabaceae, produces seeds which are characterized by seed coat dormancy (Baskin *et al*., 2000; Baskin & Baskin, 2003, 2004). Hot water immersion had a favourable effect on all treatments compared to the control and led to percentages of germination up to 56.0 ± 3.7%, for T1 treatment, being a satisfactory percentage, in the range of 60.0-79.0%, which is rated as "good" (Msanga, 1998). The germination percentage confirmed previous

studies on another taxa of *Senna* genus i.e., *S. artemisioides* ssp. × *coriacea* (Pound *et al.*, 2014), in which a heat pre-treatment of the seeds had been suggested as well. However, that research tested the seed viability by mechanical scarification, using a hand-tool (dremel tool) because of the extremely hard nature of the seed test, achieving an 82.0% percentage of seed viability, after 10 min treatment with hot water 80.0 °C. In the present study the used method involved soaking in hot water at 90.0 °C and then, the seeds were allowed to cool for 12 h resulting to soften their hard coat. The same method was effective for *Senna spectabilis* (Zembele and Ngulude, 2022) achieving germination percentages up to 67.0%, being the second from nicking treatment. The effect of the genotype in conjunction with the environmental conditions during the maturation of the seeds could explain the differences in the germination percentages. Furthermore, the effect of the environment during seed production is strong and native plants use unique strategies to adapt to their ecosystems and optimize their evolution (Baskin and Baskin, 1973; Penfield and MacGregor, 2017), influencing seed size, germination rate, viability, and variations even within the same genotype (Donohue, 2009; Donohue *et al*., 2010; Finch-savage and Bassel, 2016). Incubation of the seeds for 30 or 60 days resulted in reducing the germination rate in terms of GSI and T_{50} (Figure 3). The gradual reduction of the germination of *S. artemisioides* in the treatments indicates the possibility of the establishment of secondary lethargy.

The present study defined the lower cardinal temperature to 10.0 °C, while the germination percentage was over 30.0% at 35.0 °C (Table 1); higher upper thresholds have been found for coffee *Senna* (*Senna occidentalis*) the upper threshold of which was 45.0 °C (Norsworthy and Oliveira, 2005), while *Cassia senna* seeds also germinated over 90.0% at 40.0 °C (Al-Helal *et al.*, 1989). It could be contacted that *S. artemisioides* has upper thresholds, close to 40.0 °C, a maximum threshold for orthodox seeds (Ellis and Roberts, 1981), indicating the thermal adaptation of the species to warm environment. The present method had satisfactory results, but further research is needed to increase the germination percentages.

In vitro response of explants is dependent on culture condition and influence of plant growth regulators which can control frequency of phenotypical and physiological alterations in plants (Ziv, 1991). BA was found to be more effective than other cytokinins at stimulating axillary shoot development, while shooting was very high. Shooting percentage was very high, with no differences both to establishment and micropropagation phase and no differences as well between different media (78.0%-97.0% and 83.0%-100% respectively). Similar results were found in *Acacia mearnsii*, other member of the Fabaceae family (Feng *et al*., 1994). Increased shoot proliferation was recorded on media containing BA at ranging from 0.5-1.0 mg L^{-1}, both in the establishment and the multiplication phase and 2.4 shoots/explant formed in multiplication phase. Regarding the MI, the higher one was taken on MS containing 0.5 mg L^{-1} due to the higher shoot proliferation rate. The conjunction of 0.05 mg L^{-1} NAA with 1.0 mg L^{-1} BA did not enhance the length of formed shoots. Gladamosi and Hassan (2013) reported a low shoot number (0.3-1.1 shoots/explant) and a longer shoot length up to 3.9 cm, while they did not report shooting percentages for *Senna alata*. In comparison with the study on *in vitro* regeneration of *Senna sophera* (Parveen and Shahzad, 2014), we achieved higher shooting percentage up to 100%; on the other hand, the shoot number and length of *S. sophera* micro-shoots were higher (19.5 shoots and 5.2 cm respectively). References of other Fabaceae i.e., *Genista monosperma* (Curir *et al.*, 1986) reports similar efficiency of *in vitro* regeneration protocols.

Root formation in *in vitro* derived micro-shoots is often problematic in woody species causing considerable economic effect at this stage (De Klerk 2002; Wiszniewska *et al.,* 2016) and modified methods are often reported (Ahmad *et al.,* 2021). The rooting percentage was low in the present study. A low number of roots formed also in *S. alata* micropropagation system (Gladamosi and Hassan, 2013) with no more data of rooting percentages, while the roots in the present study grew in length twice as much. The rooting percentage was lower in comparison to *S. alata* (96.0%) in the study of Parveen and Shahzad (2014). The successful acclimatisation and survival of *in vitro* propagated plants is essential for the establishment of a successful micropropagation protocol. Several factors could interrupt the growth and lead to decline and death (Amoo *et*

al., 2011; Bunn *et al.,* 2011; Carra *et al.,* 2019). This stage is critical and the introduction of a new species in the floriculture industry as a new ornamental species is depended on the success of this. In the present study the successful acclimatisation of *S. artemisioides* was achieved like other members of *Senna* and all regenerated, rooted plantlets survived after six months and transplanted to pots.

Conclusions

The present study revealed for the first time an efficient protocol involving starting from *in vitro* grown young seedlings of *S. artemisioides* although Fabaceae are known for their recalcitrant nature. A fast and economic germination method with good seed percentages is suggested; successful establishment of initial cultures took place at high percentage on BA containing MS media. The *in vitro* multiplication rate was satisfactory, while acclimatisation rate and survivor of regenerated *S. artemisioides* was successful. The presented protocol could be used for the exploitation of *S. artemisioides* by the floriculture industry. Further studies could join the presented results to improve *in vitro* rooting efficiency and examine the possibility to establish probably an even more efficient system.

Authors' Contributions

Conceptualization: KB; Data curation: KB, KN, and PEA; Formal analysis: KB, KN and PEA; Funding acquisition: KB; Investigation: KB, KN and PEA; Methodology: KB and KN; Project administration: KB; Resources: KB, KN, PEA; Supervision: KB; Validation: KB; Visualization: KB, KN and PEA; Writing - original draft: KB; Writing - review and editing: KB. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Agrawal V, Sardar PR (2006). *In vitro* propagation of *Cassia angustifolia* through leaflet and cotyledon derived calli. Biologia Plantarum 50(1):118-122. *https://doi.org/10.1007/s10535-005-0084-8*

Ahmad A, Ahmad N, Anis M, Alatar AA, Abdel-Salam EM, Qahtan AA, Faisal M (2021). Gibberellic acid and thidiazuron promote micropropagation of an endangered woody tree (*Pterocarpus marsupium* Roxb.) using *in vitro* seedlings. Plant Cell, Tissue and Organ Culture (PCTOC) 144(2):449-462. *https://doi.org/10.1007/s11240-020-01969-1*

Ahmed MR, Ahmad N, Anis M, Aref, IM (2013). Efficient plantlet regeneration from cotyledonary node cultures of *Cassia alata* L. (Ringworm Bush): stimulatory effect of thidiazuron. Journal of Horticultural Science and Biotechnology 88:141-146.

Al-Helal AA, Al-Farraj, M M, El-Desoki RA, Al-Habashi I (1989). Germination response of *Cassia senna* L. seeds to sodium salts and temperature. Journal of the University of Kuwait(science). Kuwait 16(2):281-288.

Allan SM, Adkins SW, Preston CA, Bellairs SM (2004). Improved germination of the Australian natives: *Hibbertia commutata*, *Hibbertia amplexicaulis* (Dilleniaceae), *Chameascilla corymbosa* (Liliaceae) and *Leucopogon nutans* (Epacridaceae). Australian Journal of Botany 52:345-351. *https://doi.org/10.1071/BT03097*

Amoo SO, Finnie JF, Van Staden J (2011). The role of meta-topolins in alleviating micropropagation problems. Plant Growth Regulation 63:197-206. *https://doi.org/10.1007/s10725-010-9504-7*

Auld TD, O'Connell MA (1991). Predicting patterns of post-fire germination in 35 eastern Australian Fabaceae. Australian Journal of Ecology 16:53-70. *https://doi.org/10.1111/j.1442-9993.1991.tb01481.x*

Baskin CC, Baskin JM (1998). Seeds: ecology, biogeography, and evolution of dormancy and germination. Academic Press, San Diego, CA, pp 666.

Baskin CC, Baskin JM (2003). Classification, biogeograhpy, and phylogenetic relationships of seed dormancy. In: Smith R, Dickie J, Linington S, Pritchard H, Probert R (Eds). Seed Conservation: Turning Science into Practice. The Royal Botanic Gardens, Kew: London, pp 518-544.

Baskin J, Nan X, Baskin C (1998). A comparative study of seed dormancy and germination in an annual and a perennial species of *Senna* (Fabaceae). Seed Science Research 8(4):501-512. *https://doi.org/10.1017/S0960258500004475*

Baskin JM, Baskin CC (2004) A classification system for seed dormancy. Seed Science Research 14:1-16. *https://doi.org/101079/SSR2003150*

Baskin JM, Baskin CC, Li X (2000) Taxonomy, anatomy and evolution of physical dormancy in seeds. Plant Species Biology 15:139-152. *https://doi.org/10.1046/j.1442-1984.2000.00034.x*

Baskin JM, Baskin CC (1973). Plant population differences in dormancy and germination characteristics of seeds: heredity or environment? The American Midland Naturalist 90(2):493-498. *https://doi.org/10.2307/2424478*

Bertsouklis K, Panagaki K-P (2022). *In vitro* germination and propagation of *Dyckia brevifolia*, an ornamental and endangered Bromeliad. Horticulturae 8(5):390. *https://doi.org/10.3390/horticulturae8050390*

Bunn E, Turner SR, Dixon KW (2011). Biotechnology for saving rare and threatened flora in a biodiversity hotspot. *In vitro* Cellular and Developmental Biology-Plant 47:188-200. *https://doi.org/10.1007/s11627-011-9340-0*

Cabahug RAM, Nam SY, Lim KB, Jeon JK, Hwang YJ (2018). Propagation techniques for ornamental succulents. Korean Flower Association 26:90-101.

Carra A, Catalano C, Badalamenti O, Carimi F, Pasta S, Motisi A, (...) Garfì G (2019). Overcoming sexual sterility in conservation of endangered species: the prominent role of biotechnology in the multiplication of *Zelkova sicula* (Ulmaceae), a relict tree at the brink of extinction. Plant Cell, Tissue and Organ Culture (PCTOC) 137(1):139-148. *https://doi.org/10.1007/s11240-019-01558-x*

Carruggio F, Onofri A, Impelluso C, Giusso del Galdo G, Scopece G, Cristaudo A (2020). Seed dormancy breaking and germination in *Bituminaria basaltica* and *B. bituminosa* (Fabaceae). Plants 9(9):1110. *https://doi.org/10.3390/plants9091110*

Curir P, Damiano C, Cosmi T (1986). *In vitro* propagation of *Genista monosperma*. HortScience 21:859.

De Klerk GJ (2002). Rooting of microcuttings: theory and practice. *In Vitro* Cellular and Developmental Biolology-Plant 38:415-422. *https://doi.org/10.1079/IVP20 02335*

Donohue K (2009). Completing the cycle: Maternal effects as the missing link in plant life histories. Philosophical Transactions of the Royal Society B Biological Sciences 364:1059-1074. *https://doi.org/10.1098/rstb.2008.0291*

Donohue K, De Casas RR, Burghardt LT, Kovach K, Willis CG (2010). Germination, post germination adaptation, and species Ecological Ranges. Annual Review of Ecology, Evolution and Systematics 41:293-319. *https://www.annualreviews.org/doi/10.1146/annurev-ecolsys-102209-144715*

Ellis RH, Roberts EH (1981). The quantification of aging and survival in orthodox seeds. Seed Science and Technology 9(2):373-409.

Feng HH, Al-Khayri JM, Gbur EE (1994). Micropropagation of *Acacia mearnsii*. *In Vitro* Cellular and Developmental Biology-Plant 30:70-74.

Fett-Neto AG, Fett JP, Aquila MEA, Ferreira AG (2000). *In vitro* propagation of *Senna alata*. Planta Medica 66(02):195-196.

Finch-savage WE, Bassel GW (2016). Seed vigour and crop establishment: Extending performance beyond adaptation. Journal of Experimental Botany 67:567-591. *https://doi.org/10.1093/jxb/erv490*

Gbadamosi AE, Hassan KO (2013). *In vitro* propagation of *Senna alata* (linn) on WPM and MS media with varying PGR. International Journal of Development and Sustainability 2:1997-2007.

Generoso AL, Carvalho VS, Walter R, Campbell G, Araújo LS, Santana JGS, Cunha M (2019). Mature-embryo culture in the cryopreservation of passion fruit (*Passifora edulis* Sims) seeds. Scientia Horticulturae 256:108638. *https://doi.org/10.1016/j.scienta.2019.108638*

Holman JE, Playford J (2000). Molecular and morphological variation in the *Senna artemisioides* complex. Australian Journal of Botany 48(5):569-579. *https://doi.org/10.1071/BT98032*

International Seed Testing Association (1999). International rules for seed testing. Seed Science and Technology 27(suppl):333.

IPNI (2022). International plant names index. The Royal Botanic Gardens, Kew, Harvard University Herbaria & Libraries and Australian National Botanic Gardens. Retrieved 25 October 2022 from Published on the Internet *http://www.ipni.org*

Kwok AB, Eldridge DJ (2016). The influence of shrub species and fine-scale plant density on arthropods in a semiarid shrubland. The Rangeland Journal 38(4):381-389. *https://doi.org/10.1071/RJ15019*

Luckow M (1996). The cultivated species of *Cassia*, *Senna*, and *Chamaecrista* (Leguminosae). Baileya 23:195-241.

Maguire JD (1962). Speed of germination-Aid in selection and evaluation for seedling emergence and vigor. Crop Science 2:176-177. *https://doi.org/10.2135/cropsci1962.0011183X000200020033x*

Marazzi B, Sanderson MJ (2010). Large-scale patterns of diversification in the widespread legume genus *Senna* and the evolutionary role of extrafloral nectaries. Evolution 12:3570-3592. *https://doi.org/10.1111/j.1558-5646.2010.01086.x*.

McCown BH, Lloyd G (1981). Woody plant medium (WPM)-a revised mineral formulation for micro-culture of woody plant species. HortScience 16:453.

Msanga HP (1998). Seed germination of indigenous trees in Tanzania: Including Notes on Seed Processing, Storage and Plant Uses, Canadian Forest Service, Northern Forestry Centre (OCoLC) 41504804, Edmonton, Canada.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15(3):473-497. *https://doi.org/10.1111/j.1399-3054.1962.tb08052.x*

Norsworthy JK, Oliveira MJ (2005). Coffee senna (*Cassia occidentalis*) germination and emergence is affected by environmental factors and seeding depth. Weed Science 53(5):657-662.

Papafotiou M, Bertsouklis KF, Trigka M (2013). Micropropagation of *Arbutus unedo, A. andrachne*, and their natural hybrid, *A.* × *andrachnoides* from seedling explants. Journal of Horticultural Science and Biotechnology 6:768-775. *https://doi.org/10.1080/14620316.2013.11513037*

Papafotiou M, Martini AN, Bertsouklis KF, Vlachou G, Kanellou E, Stylias EG, Kalantzis A, ... Dariotis E (2022). A first approach for evaluation, breeding and promotion of native to Greece sage species for use as landscape plants. Acta Horticulturae 1345:359-366. *https://doi.org/10.17660/ActaHortic.2022.1345.48*

Parveen S, Shahzad A (2014). Factors affecting *in vitro* plant regeneration from cotyledonary node explant of *Senna sophera* (L.) Roxb.-A highly medicinal legume. African Journal of Biotechnology 13(3). *https://doi.org/10.5897/AJB2013.13126*

Penfield S, MacGregor DR (2017). Effects of environmental variation during seed production on seed dormancy and germination. Journal of Experimental Botany 68(4):819-825. *https://doi.org/10.1093/jxb/erw436*

Pipinis E, Hatzilazarou S, Kostas S, Bourgou S, Megdiche-Ksouri W, Ghrabi-Gammar Z, ... Krigas, N (2022). Facilitating conservation and bridging gaps for the sustainable exploitation of the Tunisian local endemic plant *Marrubium aschersonii* (Lamiaceae). Sustainability 14:1637. *https://doi.org/10.3390/su14031637*

Pound LM, Ainsley PJ, Facelli M (2014). Dormancy-breaking and germination requirements for seeds of *Acacia papyrocarpa, Acacia oswaldii* and *Senna artemisioides* ssp. *coriacea*, three Australian arid-zone Fabaceae species. Australian Journal of Botany 62(7):546-557. *http://dx.doi.org/10.1071/BT14077*

Randell BR (1989). Revision of the Cassiinae in Australia. 2. *Senna miller* sect. *Psilorhegma* (J. Vogel) Irwin & Barneby. Journal of the Adelaide Botanic Garden 12:165-272.

Randell BR, Barlow BA (1998). *Senna*. In: George AS (ex ed) Flora of Australia, vol 12. Australian Government Publishing Service, Canberra. pp 89-138.

RHS Plant Selector (2021). *Senna artemisioides*. Retrieved 2022 October 01 from *https://www.rhs.org.uk/plants/83033/senna-artemisioides/details*

Rolston MP (1978). Water impermeable seed dormancy. Botanical Review 44:365-396. *https://doi.org/10.1007/BF02957854*

Sarasan V, Kite GC, Sileshi GW, Stevenson PC (2011). Applications of phytochemical and *in vitro* techniques for reducing over-harvesting of medicinal and pesticidal plants and generating income for the rural poor. Plant Cell Reports 30:1163-1172. *https://doi.org/10.1007/s00299-011-1047-5*

Siddique I, Bukhari NAW, Perveen K, Siddiqui I (2015). Influence of plant growth regulators on *in vitro* shoot multiplication and plantlet formation in *Cassia angustifolia* Vahl. Brazilian Archives of Biology and Technology 58:686-691.

Silva SSS, Souza EH, Souza FVD, Max DAS, Rossi ML, Costa MAPC (2021). Post-seminal development and cryopreservation of endemic or endangered bromeliads. Anais da Academia Brasileira Ciências 93(1):20191133. *https://doi.org/10.1590/0001-3765202120191133*

Soltani A, Galeshi S, Zeinali E, Latifi N (2001). Genetic variation for and interrelationships among seed vigor traits in wheat from the Caspian Sea coasts of Iran. Seed Science and Technology 29:653662.

Tsoktouridis G, Krigas N, Sarropoulou V, Kampouropoulou S, Papanastasi K, Grigoriadou K, ... Maloupa E (2019). Micropropagation and molecular characterization of *Thymus sibthorpii* Benth (Lamiaceae), an aromatic-medicinal thyme with ornamental value and conservation concern. *In Vitro* Cellular & Developmental Biology-Plant 55(6):647-658. *https://doi.org/10.1007/s11627-019-10000-y*

Wiszniewska A, Nowak B, Kołton A, Sitek E, Grabski K, Dziurka M ... Tukaj Z (2016). Rooting response of *Prunus domestica* L. micro-shoots in the presence of phytoactive medium supplements. Plant Cell Tissue Organ Culture (PCTOC) 125:163-76. *https://doi.org/10.1007/s1124 0-015-0937-6*

Zembele ER, Ngulube ES (2022). Effect of seed pretreatment methods on germination and early seedling growth of *Senna spectabilis*. International Journal of Forestry Research. Article ID 6731479. *https://doi.org/10.1155/2022/6731479*

Ziv M (1991). Vitrification: morphological and physiological disorders of *in vitro* plants. In: Micropropagation. Springer Netherlands, pp 45-69.

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