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In vitro propagation and synseed mediated short-term conservation of *Scutellaria alpina* L. and *Scutellaria altissima* L.

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ABSTRACT

Nodal explants of medicinal *Scutellaria alpina* L. and *Scutellaria altissima* L. were studied for *in vitro* adventitious shoot induction, rooting, acclimatization and synseed production. Shoot induction was studied using Murashige and Skoog medium supplemented with four cytokinins individually in combination with one of the two auxins, naphthalene acetic acid or 2,4-dichlorophenoxyacetic acid. In both species, highest number of shoots were induced in the treatment containing 10 µM 6-benzylaminopurine and 0.1 µM naphthalene acetic acid. Short- term conservation of 0.2-0.3 mm long nodal explants with axillary buds was tested by forming synthetic seeds and storing at 4±2 °C for up to eight weeks. Optimization of synseed production was done by testing five concentrations of sodium alginate and two concentrations of calcium chloride, allowing 30 min for polymerization. For *S. alpina*, synseeds prepared by combining 3% sodium alginate and 100 mM calcium chloride and for *S. altissima* combining 3.5% sodium alginate and 100 mM calcium chloride resulted in 100% and 75% regeneration of explants, after three weeks of culture on Murashige and Skoog medium, respectively. Activated charcoal in the rooting medium increased root number in regenerated plants. Elongated shoots were rooted, acclimatized and successfully transferred to the greenhouse. These plants were acclimatized and established *ex vitro* and were morphologically identical to the mother plants.

Index terms: Conservation; encapsulation; medicinal plant; Skullcap; synthetic seed.

INTRODUCTION

The genus *Scutellaria* (family Lamiaceae), is used in traditional medical systems throughout Asia, Europe and North American countries (Paton, 1990; Cole et al., 2008; Shang et al., 2010; Joshee et al., 2012; Irvin et al., 2019). *Scutellaria alpina* L., (Alpine skullcap) grows in central and southern Europe and Russia and *Scutellaria altissima* L. (Somerset skullcap; tall skullcap) is a perennial herb from the mountainous regions of South Europe, East Asia and South America (Grzegorczyk-Karolak; Kuźma; Wysokińska, 2013). These two *Scutellaria* spp. are known for their medicinal properties (Grzegorczyk-Karolak; Kuźma; Wysokińska, 2013; Grzegorczyk-Karolak, Kuźma; Wysokińska, 2015a, Grzegorczyk-Karolak, Wysokińska; Olas, 2015b; Grzegorczyk-Karolak, Kuźma; Wysokińska, 2016).

Medicinal plants face considerable challenges in the propagation and regeneration due to indiscriminate harvesting and poor seed set (Canter; Thomas, Ernst, 2005; Gantait et al., 2015). There are a few reports on *in vitro* micropropagation of *Scutellaria* spp. (Joshee; Mentreddy; Yadav, 2007; Tascan et al., 2007, 2010; Cole et al., 2008; Brearley; Vaidya; Joshee, 2014; Vaidya et al., 2016) but reports on the *in vitro* propagation of *S. alpina* and *S. altissima* are limited (Grzegorczyk-Karolak; Kuźma; Wysokińska, 2016, Grzegorczyk-Karolak et al., 2017). Shoot induction studies using shoot tip explants of *S. alpina* revealed 6-Benzylaminopurine (BAP) as the most effective cytokinin (Grzegorczyk-Karolak et al., 2015a). Bioactive compounds such as baicalin, baicalein, wogonin and wogonoside reported from these species are related with medicinal properties (Gao et al., 1999; Chang; Chen; Lu, 2002; Grzegorczyk-Karolak; Kuźma; Wysokińska, 2016). Shoot and root extracts of *S. alpina* and *S. altissima* show high antioxidant activities and may have a role in the prevention of cancer, cardiovascular,

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and inflammatory diseases (Shang et al., 2010; Grzegorczyk-Karolak; Wysokińska; Olas, 2015b).

To the best of our knowledge, no research on the synseed (synthetic seed) formation and their potential in short-term low temperature conservation has been reported on these two *Scutellaria* species, so far. Benefits of synseed technology include easy handling of the tissue cultured material, limited space requirement for storage, ease of domestic and international transportation and the possibility of protection against disease and pest (Maruyama et al., 1997; Rai et al., 2009). There are reports on the successful use of this technology for herb, shrub, and tree species (Maruyama et al., 1997; Sujatha; Kumari, 2008; Lambardi; Ozudogru; Previati, 2010; Hung; Trueman, 2011; Gantait et al., 2015) and recently extending it to fern spores for mass production (Jang; Cho; Lee, 2020). Synthetic seed technology coupled with slow growth storage at 4 °C provides a viable short- to medium-term preservation strategy (Ara; Jaiswal; Jaiswal, 2000; Sharma; Shahzad; Teixeira da Silva, 2013). There are several factors ranging from initial choice of explants or plant materials, encapsulating agent, and additives that influence the success of synseed production, their storage and regeneration (Standardi; Piccioni, 1998). The objective of this study was to develop encapsulation techniques for short-term storage and distribution of two medicinal *Scutellaria* species. The study determined: (1) suitable culture media for shoot regrowth from encapsulated nodal explants, (2) short-term storage of encapsulated nodal explants at cold (4 ºC), (3) suitable storage and sowing media, (4) role of activated charcoal in plant survival and rooting, and (5) acclimatization efficiency and potting substrates for *ex vitro* plantlet production. Micropropagation derived nodal explants were used to optimize sodium alginate-calcium chloride concentrations for preparing synseeds.

MATERIAL AND METHODS

Stock culture establishment

Clean, pathogen free *in vitro* mother cultures were initiated using actively growing shoots harvested from greenhouse grown *S. alpina* and *S. altissima* plants. Shoots with five to six nodes and a shoot tip were cleaned under running tap water for 30 min and then sterilized (Joshee; Mentreddy; Yadav, 2007). Sterilized 1.0-1.5 cm long single node explants with axillary buds were inoculated in test tubes containing 15 mL basal MS medium (Murashige; Skoog, 1962; Phytotechnology Laboratories, KS, USA). Nodal and shoot tip explants were grown as sterile cultures for six to eight weeks at 25±3 °C under 16 h photoperiod. These cultures were the source of explants for all subsequent experiments.

Shoot bud induction, elongation, and rooting

MS based shoot induction medium (SIM) treatments consisted of one of the four cytokinins: 6-benzylaminopurine (BAP), *meta*-Topolin (mT), (Phytotechnology Laboratories, KS, USA), kinetin (Sigma Chemical Laboratories, MO, USA), and zeatin (Caisson Laboratories, UT, USA) at 0.1, 1.0 or 10.0 µM, in combination with auxin 0.1 µM 1- naphthalene acetic acid (NAA) (Sigma Chemical Laboratories, MO, USA) or 0.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma Chemical Laboratories, MO, USA) (Table 1).

Media treatments were adjusted to pH 5.8 prior to sterilization by autoclaving for 20 min at 121 °C (1.10 bar/15 psi). Nodal explants were inoculated in glass test tubes containing 10 mL of each shoot induction medium supplemented with 30 gL^{-1} (w/v) sucrose as a carbon source and 7 gL^{-1} (w/v) agar as the gelling agent (Carolina Biological Supply Company, NC, USA). Cultures were grown at 25±3 °C with 16 h photoperiod using cool white fluorescent tubes (Commercial Electric, Cleveland, OH, USA) at a photon flux density of 40 µmol m^{-2} s^{-1}. After three weeks in SIM treatments, cultures were transferred to 15 mL elongation medium (basal MS medium with 30 gL^{-1} sucrose and 7 gL^{-1} agar) in test tubes at culture room conditions. Shoot induction response of nodal explant was calculated by destructive counting of cultures after four weeks of transfer to the elongation medium.

Synseed production

Experiments were carried out to optimize combination of sodium alginate and calcium chloride concentrations to make synseeds with proper strength and shape to encapsulate nodal explants. Sodium alginate (Phytotechnology Laboratories, KS, USA) at 2, 2.5, 3, 3.5 and 4% (w/v) as gelling agent and calcium chloride (Sigma Chemicals, MO, USA) as complexing

agent was used at 75 mM and 100 mM. Sodium alginate solutions were prepared in basal MS medium, whereas calcium chloride solutions were prepared in autoclaved distilled water. Basal MS medium contains calcium chloride ($CaCl_2.2H_2O$; m.w. 147.01) at 440 mgL^{-1} that adds 2.99 mM to 75 and 100 mM treatments. All solutions were sterilized by autoclaving at 121 °C (1.10 bar/15 psi) for 15 min and calcium chloride solutions were adjusted to a pH of 5.8 prior to autoclaving. Nodal segments obtained from micropropagated stock plants were trimmed into 2.0 - 3.0 mm segments with two axillary buds for encapsulation. These nodal explants were suspended in the sodium alginate solution in a Petri dish (Fisher Scientific, MA, USA) and then gently dropped into calcium chloride solution with the help of a dropper and left for 30 min to achieve complete polymerization. Polymerized alginate beads (synseeds) were rinsed in autoclaved distilled water three times to remove excess calcium chloride, and then transferred to sterilized paper towel to remove excess moisture. Synseeds produced using various combinations of sodium alginate and calcium chloride concentrations were visually recorded for their shape morphology and color. To study the effect of synseed strength on explant regeneration, freshly prepared 20 synseeds for each treatment were transferred to MS basal medium for three weeks in culture room conditions to germinate and grow.

Table 1: Murashige and Skoog Medium based shoot induction medium treatments for the micropropagation of *S. alpina* and *S. altissima*.

Treatment #	BAP (µM)	m-Topolin (µM)	Kinetin (µM)	Zeatin (µM)	2,4-D (µM)	NAA (µM)
1.	-	-	-	-	-	-
2.	0.1				0.5	
3.	1.0				0.5	
4.	10.0				0.5	
5.	0.1					0.1
6.	1.0					0.1
7.	10.0					0.1
8.		0.1			0.5	
9.		1.0			0.5	
10.		10.0			0.5	
11.		0.1				0.1
12.		1.0				0.1
13.		10.0				0.1
14.			0.1		0.5	
15.			1.0		0.5	
16.			10.0		0.5	
17.			0.1			0.1
18.			1.0			0.1
19.			10.0			0.1
20.				0.1	0.5	
21.				1.0	0.5	
22.				10.0	0.5	
23.				0.1		0.1
24.				1.0		0.1
25.				10.0		0.1

Effect of low temperature and storage medium on regeneration

This experiment evaluated the germination ability of synseeds that were prepared by 3% sodium alginate and 100 mM calcium chloride and stored at 4 ±2 °C. Five time points (0, 2, 4, 6 and 8 weeks) were selected to evaluate regeneration of cold stored synseeds and 20 synseeds were tested for each time point for both species. After encapsulating nodal segments, two replicates of 100 synseeds each, were transferred to Petri dishes containing 1 mL sterile distilled water to maintain humidity and stored at 4 ±2 °C. At each time point, 20 synseeds were transferred to basal MS medium and incubated in culture room for three weeks to score regeneration. In another experiment, 20 synseeds for each treatment were stored at 4 ±2 °C in three different semi-solid MS media compositions (storage medium): full strength MS medium, full strength MS with 2 µM BAP and full-strength MS with 2 µM BAP and 0.2 µM NAA to study the role of plant growth regulators on the regrowth of encapsulated *Scutellaria* explants. Thus, for each time point there were three treatments for each species. After each cold storage period, 20 synseeds were removed and placed on basal MS medium for germination in culture room conditions. After three weeks, regrowth in synseeds was recorded.

Effect of sowing medium formulation on regeneration

Synseeds (3% sodium alginate + 100 mM calcium chloride) were stored in a petri dish at 4 ±2 °C for two weeks with 1 mL sterile water. After two weeks of cold storage, 20 beads were sown on each sowing media to investigate the role of different MS medium based formulations on germination and further growth. Six semi-solid MS medium based sowing media as following compositions were tested: half strength MS medium, half strength MS medium with 2 µM BAP, half strength MS medium with 2 µM BAP and 0.1 µM NAA, full strength MS medium, full strength MS medium with 2 µM BAP, and full-strength MS medium with 2 µM BAP and 0.1 µM NAA. Germination percentage, shoot length, and shoot number was recorded after three weeks of transfer to the sowing medium.

Addition of activated charcoal in the rooting medium

Half strength MS medium was supplemented with 0.1 and 0.2 gL^{-1} activated charcoal in addition to auxin 0.1 µM NAA and BAP at 2.0 µM, individually or in combination for binding detrimental polyphenols and stimulating root development. All treatments contained 30 gL^{-1} of sucrose and 7 gL^{-1} agar. Prior to dispensing 15 mL/culture tube, all media were adjusted to pH 5.8 and autoclaved at 121 °C for 20 min. This experiment used synseeds prepared using optimized formulation of 3% (w/v) sodium alginate and 100 mM calcium chloride. After initial two weeks at 4 ±2 °C, synseeds were transferred to seven treatments to test effect of activated charcoal on rooting after six weeks of growth at culture room conditions.

Acclimatization

Well-developed shoots regenerating from synseeds were rooted in half strength MS medium. Plants with multiple adventitious roots were removed from the test tubes, gently washed under tap water to remove agar and media constituents. Plants were transferred to two magenta box assembly containing about 65 g sterile potting mix (PROMIX BX, PA, USA) with half strength sterile MS medium. These boxes with three to four *in vitro* rooted plants were placed in the culture room for acclimatization. After a week, upper box was gently opened forming a crack to reduce humidity and after two weeks it was completely removed. Four weeks later hardened plants with new growth were transferred to larger pots containing potting mix and perlite (3:1) moistened with tap water and kept in the greenhouse for further growth.

Statistics and data analysis

All experiments were repeated three times with 20 nodal segments for each treatment for both species. Data was recorded on regeneration and germination percentage, number of shoots per explant, and average shoot length (cm). The regeneration frequencies were calculated as the percentage of encapsulated nodal segment inoculated in a particular treatment. Analysis of the data was carried out using Analysis of Variance (ANOVA) and the differences in treatments means was compared using Tukey's test least significance difference (LSD) at 0.5% level of significance (Steel; Torrie; Dickey, 1980).

RESULTS AND DISCUSSION

Establishment of stock cultures and shoot organogenesis

Scutellaria alpina and *S. altissima* responded by axillary bud break within 5-10 days after inoculation in shoot induction media treatments (Table 1). There were differences in the number of adventitious shoot buds after three weeks of incubation in SIM treatments. After three weeks on the SIM, explants were transferred to basal MS medium for the elongation of shoot buds for three more weeks and then shoot counts were performed (Table 2, Figure 1A). As shown in Table 2, 10.0 µM BAP + 0.1 µM NAA treatment produced highest number of shoots for both *Scutellaria* species. MS basal medium as the control treatment did not produce any adventitious shoots. The maximum mean harvestable shoot number was 6 and 5.2 for *S. alpina* and *S. altissima* in response to the treatment 10.0 µM BAP + 0.1 µM NAA, respectively (Figure 1A; Table 2). Inclusion of 0.5 µM 2, 4–D, in addition to cytokinins was detrimental to the shoot induction process as it favored callus production (Table 2). This result is in agreement with Joshee et al. (2007) where highest *in vitro* shoot proliferation from the nodal explants of *Scutellaria integrifolia* was observed on MS medium supplemented with 2.2 µM BAP and 0.54 µM NAA. Similar results were found in *Etlingera elatior* where shoot regeneration was best supported on MS medium supplemented with 13.32 µM BAP (Yunus et al., 2012). Two SIM treatments, 0.1 µM kinetin with 0.1 µM NAA and 0.1 µM mT with 0.1 µM NAA recorded the highest mean shoot length (13.8 ± 0.2 cm) and (14.0 ± 0.63 cm) for *S. alpina* and *S. altissima,* respectively (data not presented). A positive role of m-Topolin in the adventitious shoot bud induction is reported on *S. barbata* and *S. racemosa* (Brearley; Vaidya; Joshee, 2014). The effects of various cytokinins on bud break has also been reported for *Ceropegia bulbosa* var. *bulbosa*, 8.88 µM BAP being most effective (Dhir; Shekhawat, 2013). The result also indicates that in *S. alpina*, m-Topolin and kinetin induce shoot regeneration when combined with NAA (Table 2). As expected, when cytokinins were combined with 0.5 µM 2,4-D, poor shoot induction accompanied by callusing at the base of the explants was observed and continued exposure resulted in necrosis leading to death.

Table 2: Shoot induction response of *S. alpina* and *S. altissima* nodal explants on various shoot induction media for three weeks and further growth on elongation medium for four weeks.

#	Treatments (µM)	*S. alpina*	*S. altissma*
1	Control (MS basal)	1.0 ± 0.18^{d}	1.0 ± 0.0^{d}
2	0.1 µM BAP + 0.5 µM 2,4-D	1.2 ± 0.18^{d}	1.2 ± 0.18^{d}
3	1.0 µM BAP + 0.5 µM 2,4-D	1.2 ± 0.0^{d}	1.0 ± 0.0^{d}
4	10.0 µM BAP + 0.5 µM 2,4-D	1.0 ± 0.22^{d}	1.0 ± 0.0^{d}
5	0.1 µM BAP + 0.1 µM NAA	2.4 ± 0.73^{d}	2.2 ± 0.52^{b}
6	1.0 µM BAP + 0.1 µM NAA	5.4 ± 0.63^{ab}	2.0 ± 0.0^{c}
7	10.0 µM BAP + 0.1µM NAA	6.0 ± 0.22^{a}	5.2 ± 1.21^{a}
8	0.1µM KN + 0.5 µM 2,4-D	1.6 ± 0.36^{c}	1.2 ± 0.0^{d}
9	1.0 µM KN + 0.5 µM 2,4-D	1.8 ± 0.0^{c}	1.0 ± 0.0^{d}
10	10.0 µM KN + 0.5 µM 2,4-D	1.2 ± 0.18^{d}	1.4 ± 0.22^{c}
11	0.1 KN µM + 0.1 µM NAA	3.0 ± 0.63^{c}	2.6 ± 0.36^{b}
12	1.0 µM KN + 0.1 µM NAA	1.8 ± 0.72^{c}	2.2 ± 0.22^{b}
13	10.0 µM KN + 0.1 µM NAA	3.4 ± 0.22^{c}	2.8 ± 0.40^{b}
14	0.1 µM mT + 0.5 µM 2,4-D	1.4 ± 0.18c^{d}	1.0 ± 0.18^{d}
15	1.0 µM mT + 0.5 µM 2,4-D	1.4 ± 0.18^{d}	1.0 ± 0.0^{d}
16	10.0 µM mT + 0.5 µM 2,4-D	1.0 ± 0.40^{d}	1.4 ± 0.22^{c}
17	0.1 µM mT + 0.1 µM NAA	1.2 ± 0.33^{d}	1.6 ± 0.78^{c}
18	1.0 µM mT + 0.1 µM NAA	3.0 ± 0.46^{c}	1.6 ± 0.18^{c}
19	10.0 µM mT + 0.1 µM NAA	4.8 ± 0.22^{b}	2.0 ± 0.59^{c}
20	0.1 µM ZT + 0.5 µM 2,4-D	1.4 ± 0.18^{d}	1.4 ± 0.22^{c}
21	1.0 µM ZT + 0.5 µM 2,4-D	1.8 ± 0.0^{c}	1.4 ± 0.22^{c}
22	10.0 µM ZT + 0.5 µM 2,4-D	1.0 ± 0.0^{d}	1.0 ± 0.0^{d}
23	0.1 µM ZT + 0.1 µM NAA	1.0 ± 0.44^{d}	1.2 ± 0.18^{d}
24	1.0 µM ZT + 0.1 µM NAA	2.8 ± 0.44^{c}	1.8 ± 0.52^{c}
25	10.0 µM ZT + 0.1 µM NAA	2.2 ± 0.0^{d}	1.8 ± 0.52^{c}

Each number represent means shoot number with ± SE of five replicate per treatment. Means follow by the same values are not significantly different according to Tukey's test ($P \leq 0.05$). The results obtained for two species were not compared with each other during statistical analysis.

Synseed production, quality characterization, and regrowth/ regeneration

Five concentration of sodium alginate (2.0, 2.5, 3.0, 3.5, and 4.0% w/v) were tested with two concentrations of calcium chloride (75 and 100 mM) as complexation agent to formulate alginate beads for

encapsulation that will be suitable for assessing cold preservation and thereafter regeneration of plants for greenhouse/field transfer. A 30 min complexation reaction was allowed for all experiments. Sodium alginate concentration had an effect on the shape, strength, and color of synseeds containing nodal segments of *S. alpina* and *S. altissima.* Differences in the morphology of beads with respect to their shape, texture, strength and transparency were evident. In this experiment, 3% sodium alginate polymerized using 100 mM calcium chloride was the best treatment resulting in the formation of firm, clear and isodiametric synseeds that allowed shoot and root regrowth (Figure 1B, C; Table 3). The synseeds formed using 2 and 2.5% sodium alginate with 75- and 100 mM calcium chloride concentrations were soft, fragile, and irregular in shape. However, when the sodium alginate concentration was 3, 3.5 or 4%, the synseeds formed were isodiametric and morphologically stable (Figure 1B-D). Similar results were seen on the encapsulation of shoot tips of *Psidium guajava* L. (Rai et al., 2009) and *Withania somnifera* L. (Fatima et al., 2013). Sodium alginate provides protection to the tissue and strength to the synseeds enabling easy handling and transportation. Among the sodium alginate concentration tested, the highest germination percentage (100%) for *S. alpina* was obtained in synseeds formed using 3% sodium alginate.

The synseeds produced with lower sodium alginate concentration (2.0 and 2.5%) could not polymerize well to provide proper protection to the encapsulated tissue during handling and inoculation. Sodium alginate concentration is an important factor that facilitates an optimum exchange between Ca^{++}

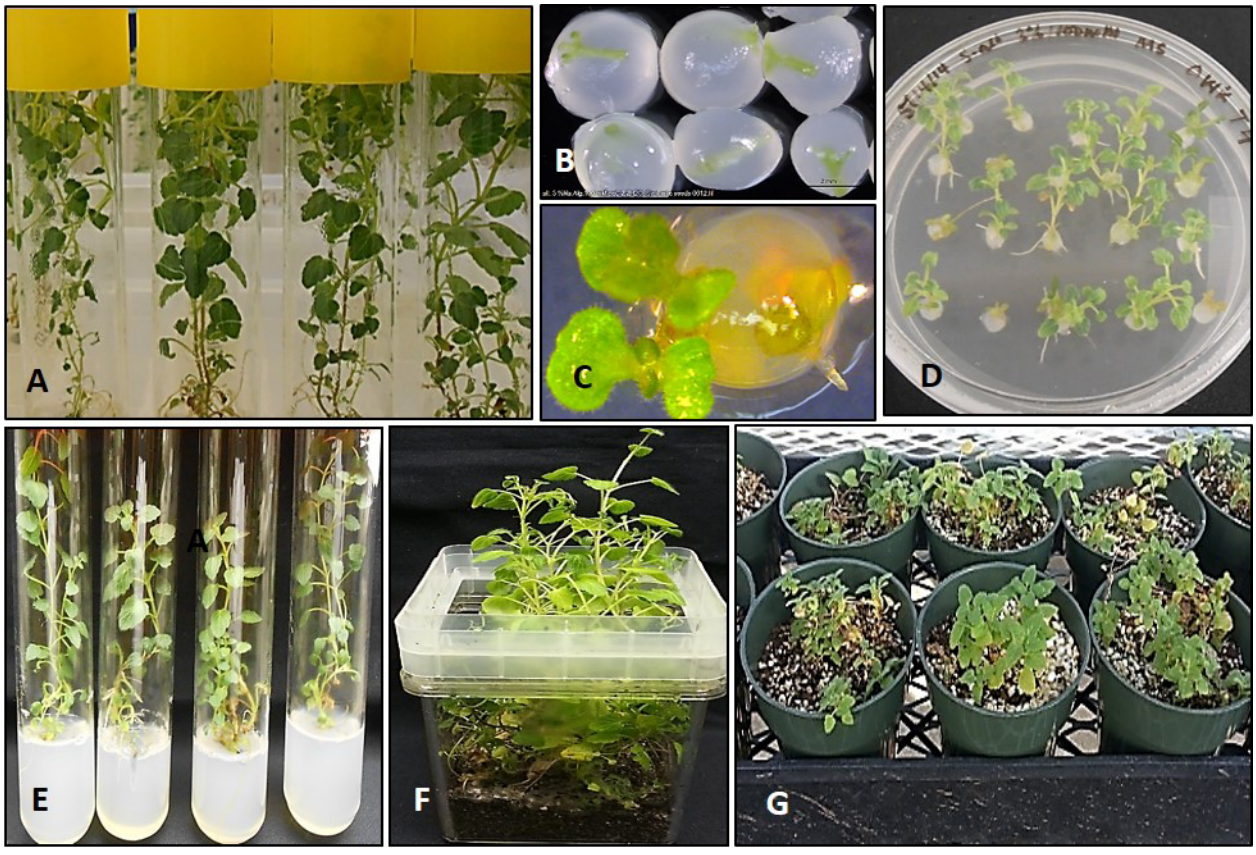

Figure 1: Micropropagation and encapsulation studies on *Scutellaria alpina* and *Scutellaria altissima.* (A), Micropropagation using nodal explants (B), Nodal explants encapsulated in synseeds prepared with 3% sodium alginate and 100mM calcium chloride solution (C), Two weeks old regenerated synthetic bead (D), Successful germination of synthetic seeds prepared with 3% sodium alginate and 100 mM calcium chloride (E), Regenerated nodal explants from synseeds growing in the culture tubes (F), Regenerated plants in magenta box containing sterile soil mix for acclimatization (G), Plantlets growing in pots containing potting mix and perlite 3:1 regenerated from synseeds.

and Na^+ ions for polymerization to occur (Bekheet; Taha; El-Bah, 2005). These results indicate that there is an increase in the germination potential of synseeds when the concentration of sodium alginate is in the range of 3.0-4.0% (Figure 1C).

Similar conclusion was also deduced on a medicinal herb *Spilanthes acmella* where synseeds prepared by 3% sodium alginate and 100 mM calcium chloride treatment encapsulating shoot tips exhibited best results (Singh et al., 2009). However, synseeds produced with 4% sodium alginate resulted in lower germination, especially in case of *S. altissima*, that can be attributed to the rigid and hard nature of the beads, leading to delay or no sprouting (Fatima et al., 2013). As evident, 3% sodium alginate and 100 mM calcium chloride combination proved ideal for making synseeds with desirable characteristics for *Scutellaria* spp. (Table 4; Figure 1D). Two additional lower calcium chloride concentrations (25 mM or 50 mM) were also tested for polymerization but required longer polymerization period and produced unsatisfactory synseeds (data not presented).

Table 3: Effects of different concentrations of sodium alginate and two concentration of calcium chloride on quality characteristics of encapsulated synseeds.

#	Sodium alginate (w/v %)	Calcium chloride (mM)	Synseed Morphology	Color	Quality
1	2.0	75	Shapeless and loose	Transparent	**
2	2.0	100	Fragile and soft	Transparent	**
3	2.5	75	Fragile and soft	Transparent	**
4	2.5	100	Fragile and soft	Transparent	**
5	3.0	75	Isodiametric and compact	Transparent	**
6	3.0	100	Isodiametric and compact	Transparent/white	****
7	3.5	75	Isodiametric and compact	Transparent/white	***
8	3.5	100	Isodiametric and compact	Transparent/white	***
9	4.0	75	Isodiametric and hard	Transparent/white	***
10	4.0	100	Isodiametric and hard	White	***

****(Excellent form beads), (2) ***(Good beads), (3) **(Poorly form beads) complexed with100 mM calcium chloride and for *S. altissima* it was 75% recorded in 3% sodium alginate complexed with 75 mM calcium chloride and 3.5% sodium alginate in combination with 100 mM calcium chloride, both. Lowest germination for *S. alpina* was 35% with the synseeds prepared with 2% sodium alginate and 75 mM calcium chloride while for *S. altissima* it was 13.3% for synseeds prepared with 3.5% sodium alginate complexed with 75 mM calcium chloride (Table 4).

Table 4: *In vitro* germination efficiency of *S. alpina* and *S. altissima* synseeds without cold preservation after three weeks of incubation on MS medium in culture room conditions.

#	Treatments	*S. alpina*	*S. altissima*
1	2% Sodium alginate + 75 mM $CaCl_2.2H_20$	35.0 ± 4.08^{d}	16.67 ± 2.72^{e}
2	2% Sodium alginate + 100 mM $CaCl_2.2H_20$	43.34 ± 3.60^{d}	48.33 ± 4.90^{c}
3	2.5% Sodium alginate + 75 mM $CaCl_2.2H_20$	53.34 ± 1.36^{c}	51.67 ± 3.60^{b}
4	2.5% Sodium alginate + 100 mM $CaCl_2.2H_20$	61.67 ± 1.36^{c}	65.0 ± 6.24^{a}
5	3% Sodium alginate + 75 mM $CaCl_2.2H_20$	91.67 ± 1.36^{a}	73.33 ± 5.93^{a}
6	3% Sodium alginate + 100 mM $CaCl_2.2H_20$	100.0 ± 0.0^{a}	61.33 ± 19.90^{b}
7	3.5% Sodium alginate + 75 mM $CaCl_2.2H_20$	42.0 ± 21.64^{d}	13.33 ± 1.08^{e}
8	3.5% Sodium alginate + 100 mM $CaCl_2.2H_20$	76.67 ± 2.72^{b}	75.0 ± 4.08^{a}
9	4% Sodium alginate + 75 mM $CaCl_2.2H_20$	73.34 ± 5.44^{b}	48.33 ± 2.72^{c}
10	4% Sodium alginate + 100 mM $CaCl_2.2H_20$	70.0 ± 6.24^{b}	33.33 ± 3.60^{d}

The means followed by the same letters for the same species are not significantly different according to Tukey's test ($P \leq 0.05$). Each number represent means ± SE of 20 replicates per treatment in three repeated experiments. The results obtained for two species were not compared during statistical analysis.

Cold preservation duration and regrowth of encapsulated explant

Synseeds (3% sodium alginate + 100mM calcium chloride) stored at 4 °C ± 2 °C for a period of 0, 2, 4, 6 and 8 weeks were evaluated to determine effects of cold storage on regeneration potential of encapsulated nodal explants. It was observed that synseeds of *S. alpina* and *S. altissima* could be stored for up to four weeks without substantial decrease in their regeneration potential (Table 5). These results indicate that the synseed germination for *S. alpina* and *S. altissima* was 61.67% and 81.67% after four weeks of storage in autoclaved distilled water, respectively. Gradual decrease in the synseed germination was observed for subsequent weeks dropping to 31.67% for *S. alpina* and 48.33% for *S. altissima* after eight weeks (Table 5). Similar results were observed in encapsulation of *Corymbia torelliana* x *C. citriodora* (Hung; Trueman, 2012), shoot of *Olea europaea* L. (Micheli; Hafiz; Standardi, 2007) and shoot tips of *Solanum nigrum* (Verma et al., 2010).

Synseed regrowth varies considerably depending on the genotype, explant and cold duration as was seen in case of plantation eucalypt after 8 weeks storage at 4 °C, 0 -12% regrowth was observed (Hung; Trueman, 2012). The reduction in regrowth is attributed to the inhibition of respiration due to encapsulation matrix, desiccation due to loss of water by the tissue or both.

Table 5: Effects of cold storage on *in vitro* germination of *S. alpina* and *S. altissima* synseeds after 0-8 weeks of cold storage and then three weeks of growth on basal MS medium in the culture room.

#	Weeks	Regrowth % for *S. alpina*	Regrowth % for *S. altissima*
1	0	81.67 ± 1.36^{a}	100,00 ± 0^{a}
2	2	75.00 ± 2.35^{a}	95.00 ± 2.36^{a}
3	4	61.67 ± 1.36^{b}	81.67 ± 1.36^{b}
4	6	43.33 ± 1.36^{c}	66.67 ± 2.72^{c}
5	8	31.67 ± 3.60^{d}	48.33 ± 3.60^{d}

Each value represent means ± SE of 20 replicates per treatment in three repeated experiment. The means followed by the same letters for the same species are not significantly different according to Tukey's test ($P \leq 0.05$) and the results were not compared in statistical analysis.

Effects of storage media on plantlet development

Scutellaria alpina and *S. altissima* synseeds (3% sodium alginate + 100 mM calcium chloride) exhibited varied responses in regrowth when stored at 4 ± 2 ºC for 0, 2, 4, 6, and 8 weeks using three compositionally different storage media (Table 6).

The highest synseed regrowth was 86.67% for *S. alpina* and 98.33% for *S. altissima* when synthetic seeds were stored in MS supplemented with 2.0 µM BAP and 0.2 µM NAA without cold storage among all time points and subsequently germinated in semi-solid MS medium within a week. Decline in percentage regrowth was observed in *S. alpina* at 43.33% and in *S. altissima* at 50% when it was stored in MS without PGRs up to eight weeks and regenerated in semi-solid MS medium that improved to 55 and 61.67% by including growth regulators (Table 6). The higher germination of the synthetic seed after a short-term storage is an indication that, during the storage period, encapsulated tissues were absorbing some of the nutrients contained in the media that enabled it to retain moisture preventing quick desiccation. *Citrus reticulata* somatic embryos that were encapsulated with an artificial endosperm containing GA_3 was useful for the storage of synseeds (Antonietta; Manuel; Alvaro, 1998). Therefore, the stored synseeds are able to imbibe some of the nutrients in the media during the storage period. In general, synseeds stored in liquid MS with plant growth regulators demonstrate higher level of germination than those stored in MS without PGRs suggesting significant role synseeds may play in short term germplasm conservation (Table 6).

Effects of sowing media and plant growth regulators on regrowth of synseeds

Compositionally different media (half- and full- strength basal MS and with BAP alone or BAP + NAA) were examined for regrowth and conversion into shoots from synseed (3% sodium alginate and 100 mM calcium chloride) that were cold stored for two weeks. Among the different sowing media treatments tested, the highest synseed germination was obtained in MS + 2 µM BAP + 0.2 µM NAA with 88.33% for *S. alpina* and 95% was for *S. altissima*. The lowest frequency of germination was 78.33% and 76.66% in half

MS without any plant growth regulators treatment for *S. alpina* and *S. altissima*. After three weeks of growth, longest shoot length was 7.1 cm for *S. alpina* in half strength MS medium and 9.6 cm for *S. altissima* in MS + 2 µM BAP + 0.2 µM NAA treatment, respectively (Figure 1E; Table 7). Half strength MS medium supplemented with 2 µM BAP and 0.2 µM NAA recorded average shoot length of 3.2 cm for *S. alpina* and 4.7 cm for *S. altissima*. Highest mean shoot numbers of 4.6 and 3.8 were recorded in the treatment MS supplemented with 2 µM BAP (Table 7). The minimum mean shoot number was also obtained from media devoid of plant growth regulators. Earlier studies have suggested that the medium, PGR concentrations and genotype of the plant contributes as important factors in elongation and proliferation of shoots (Pati et al., 2006; Nhut; Hai; Phan, 2010). Interaction between various cytokinins and auxins are considered to be one of the most important factors in growth and development in plant tissue culture (Gasper et al., 1996; Moubayidin; Di Mambro; Sabatini, 2009), however, the plant species play a vital role as revealed by two species in this experiment.

Effects of activated charcoal on rooting

After three weeks of germination on MS basal medium, well-developed 2-3 nodes long shoots of *S. alpina* and *S. altissima* were harvested for root induction. Root induction was tested in half strength MS medium supplemented with 0.1-0.2 gL^{-1} activated charcoal, plant growth regulators (NAA and BAP), 30 gL^{-1} sucrose and 7 gL^{-1} agar (Table 8). Rooting response was evaluated after six weeks of transfer. All treatments produced more roots than the control (half strength MS without PGRs or activated charcoal). There was high rooting of encapsulated nodes in the treatment where half strength MS medium contained 0.1 NAA with 0.2 gL^{-1} activated charcoal. The average root number of 7.8 and 9.8 per synseed was recorded for *S. alpina* and *S. altissima*, respectively (Table 8). Morphologically, roots in activated charcoal containing media were thicker and longer. Study on *Swertia chirayita* is in agreement with our results as addition of 0.5 gL^{-1} activated charcoal enhanced root number (Joshi; Dhawan, 2007). Similar observation were made during *in vitro* micropropagation of *Fortunella*

Table 6: Effects of three storage medium composition on *in vitro* germination of 0-8 weeks cold stored synseeds of *S. alpina* and *S. altissima* upon transfer to basal MS medium for three weeks.

Week	Media composition	*S. alpina*	*S. altissima*
0	MS basal	68.33 ± 3.60^{b}	85.00 ± 2.36^{a}
	MS + 2.0 µM BAP	85.00 ± 2.36^{a}	93.33 ± 1.36^{a}
	MS + 2.0 µM BAP + 0.2 µM NAA	86.67 ± 1.36^{a}	98.33 ± 1.36^{a}
2	MS basal	65.00 ± 2.36^{b}	68.33 ± 1.36^{b}
	MS + 2.0 µM BAP	80.00 ± 2.36^{a}	91.67 ± 1.36^{a}
	MS + 2.0 µM BAP + 0.2 µM NAA	81.67 ± 1.36^{a}	96.67 ± 2.72^{a}
4	MS basal	61.67 ± 1.36^{b}	71.67 ± 1.36^{b}
	MS + 2.0 µM BAP	75.00 ±2.36^{a}	88.33 ± 3.60^{a}
	MS + 2.0 µM BAP + 0.2 µM NAA	78.33 ± 3.60^{a}	88.33 ± 3.60^{a}
6	MS basal	43.33 ± 1.36^{c}	63.33 ± 2.72^{b}
	MS + 2.0 µM BAP	66.67 ± 2.72^{b}	65.00 ± 2.36^{b}
	MS + 2.0 µM BAP + 0.2 µM NAA	61.67 ± 2.72^{b}	71.67 ± 2.72^{b}
8	MS basal	43.33 ± 1.36^{c}	50.00 ± 2.36^{c}
	MS + 2.0 µM BAP	55.00 ±2.36^{b}	55.00 ± 2.36^{c}
	MS + 2.0 µM BAP + 0.2 µM NAA	51.67 ± 1.36^{c}	61.67 ± 1.36^{b}

Each number represents means ± standard error of 20 synseeds per treatment in three repeated experiments. First column denotes weeks of storage at 4 ± 2 ºC. The means followed by the same letter with the same species are not significantly different according to Tukey's test ($P \leq 0.05$). The results obtained for two species were not compared during statistical analysis.

crassifolia in activated charcoal supplemented medium (Yang et al., 2006). Addition of 0.05 gL^{-1} activated charcoal to the MS medium improved rooting and minimized callus during *in vitro* regeneration of *Simmondsia chinensis* (Agrawal; Prakash; Gupta, 2002).

Acclimatization

Survival of the plants in the substrate moistened with half strength MS or autoclave distilled water were in the range of 85 to 90% for both species. Plants did not show any detectable variations with respect to its morphology (Figure 1F, G). This finding is in agreement with the research done on *Rauvolfia serpentina* (Faisal et al., 2012), *Rauvolfia tetraphylla* (Alatar; Faisal, 2012), nodal segments of *Punica granatum* (Naik; Chand, 2006), and shoot tips of *Phyllanthus amarus* (Singh et al., 2006).

Current study indicates the potential of synthetic seed technology in *ex situ* conservation of two *Scutellaria* species. Large numbers of nodes can be harvested through an efficient *in vitro* protocol development. Positive role of inclusion of growth regulators in storage and sowing medium was evident in shoot multiplication, regrowth, and survival (Table 6, 7). It would be interesting to test osmotic regulants and other cryoprotectants like glycerol, dimethyl sulfoxide (DMSO) as additives in combination with growth regulators to increase shelf life of synseeds during cold storage. More research is required on using other explants like shoot tip for their longer survival during cold preservation.

Table 7: Effects of sowing media composition on *in vitro* regrowth, shoot length and shoot number of *S. alpina* and *S. altissima* synseeds.

Treatments	Explant regrowth %		Shoot Length (cm)		Shoot Number	
	S. alpina	*S. altissima*	*S. alpina*	*S. altissima*	*S. alpina*	*S. altissima*
½ MS	78.33 ± 1.7	76.66 ± 4.4	7.1 ± 0.4	8.1 ± 0.5	2.0 ± 0.0	2.0 ± 0.0
½ MS + 2 µM BAP	80.00 ± 7.6	85.00 ±7.6	4.2 ± 0.7	6.1 ± 0.7	3.2 ± 0.3	3.2 ± 0.4
½ MS + 2 µM BAP + 0.2 µM NAA	78.33 ± 7.6	81.66 ± 6.0	3.2 ± 0.4	4.7 ± 0.3	2.4 ± 0.8	3.6 ± 0.3
MS	80.00 ± 7.6	93.33 ± 1.7	5.5 ± 1.0	8.4 ± 0.2	1.6 ± 0.2	2.0 ± 0.0
MS + 2 µM BAP	85.00 ± 2.9	93.33 ± 3.33	6.2 ± 0.7	8.7 ± 0.4	4.6 ± 0.3	3.8 ± 0.5
MS + 2 µM BAP + 0.2 µM NAA	88.33 ± 1.6	95.00 ± 2.9	7.0 ± 0.9	9.6 ± 0.4	2.0 ± 0.2	3.4 ± 0.3

Values represent means ± SE of 20 synseeds per treatment in three repeated experiments for germination and five replicates of three repeated experiments for shoot length and shoot number experiments.

Table 8: Effects of activated charcoal alone or in combination with plant growth regulators on root induction in *S. alpina* and *S altissima*.

Media Composition	Number of Roots/synseed	
	S. alpina	*S. altissima*
½ MS	3.4 ± 0.24^{b}	3.6 ± 0.24^{c}
½ MS + 0.1g L^{-1} AC	6.0 ± 0.31^{ab}	5.8 ± 0.37^{bc}
½ MS + 0.2g L^{-1} AC	6.8 ± 0.73^{a}	9.2 ± 0.58^{ab}
½ MS + 0.1 µM NAA + 0.1g L^{-1} AC	6.6 ± 0.40^{ab}	6.4 ± 0.24^{b}
½ MS + 0.1 µM NAA + 0.2g L^{-1} AC	7.8 ± 0.86^{a}	9.8 ± 0.86^{ba}
½ MS + 0.1 µM NAA + 2.0 µM BAP + 0.1g L^{-1} AC	4.4 ± 0.54^{b}	6.4 ± 0.67^{b}
½ MS + 0.1 µM NAA + 2.0 µM BAP + 0.2g L^{-1} AC	6.0 ± 0.54^{ab}	7.0 ± 0.83^{b}

Values represent means root number ± SE of three repeated experiments. Means follow by the same letters are not significantly different according to Tukey's test ($P \leq 0.05$). The results obtained for two species were not compared during statistical analysis. AC: activated charcoal.

CONCLUSIONS

Synseeds (artificial/synthetic seed) have been produced by encapsulation of somatic embryos, shoot buds, shoot tips, calli, nodal segments, embryogenic masses, protocorms and protocorm like bodies with specific coating matrix that provides protection and nutrition to the encapsulated propagule. Since, somatic embryogenesis has not yet been reported in these species, encapsulation of nodes to develop synthetic seeds could be a suitable alternative for conservation. A reliable and efficient protocol has been developed for micropropagation using nodal explants, synseed formation and germination, cold preservation, conversion, and acclimatization leading to greenhouse transfer and thus assisting short-term storage of two medicinal *Scutellaria* species. Nodal segments with two axillary buds proved to be an ideal propagule for *S. alpina* and *S. altissima* that can be successfully used for short -term cold preservation using synseed technology. *In vitro* propagation and multiplication can be further scaled up by using shoot tip that will bring down cost of production. This study will further help our efforts in producing synthetic seeds to assist our conservation efforts related to rare and threatened *Scutellaria* germplasm in our collection. The high frequency of plantlet survival and transition to greenhouse conditions from encapsulated nodal segments of *Scutellaria* species after 8 weeks of storage at low temperature could be used as a delivery system for germplasm exchange, and offers the possibility of using this method for *ex situ* conservation.

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