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Cytokinin, Carbon Source, and Acclimatization Requirements for *in Vitro* Propagation of *Scutellaria barbata* D. Don and *Scutellaria racemosa* Pers.

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Abstract

Micropropagation protocols to minimize hyperhydricity were optimized for medicinal *Scutellaria barbata* and *Scutellaria racemosa*. Six cytokinins and eight different carbon sources at two different incubation periods of 14 and 21 days were studied for adventitious shoot bud induction using nodal explants. In *S. barbata*, 5 µM *meta*-Topolin and 0.1 µM NAA supplemented shoot induction medium produced four shoots each after 14 and 21 day incubation. Observation of *S. racemosa* nodal explants recorded four and five shoots after 14 and 21 day incubation. In both species, control explants (no plant growth regulators in the medium) consistently resulted in the bud break with two shoots in both 14 and 21 day incubation. The effect of carbon source on shoot regeneration was studied by supplementing eight different sugars at 0.1 M concentration to the optimized shoot induction medium (5 µM *meta*-Topolin and 0.1 µM NAA). *S. barbata* nodal explants cultured on shoot induction medium supplemented with fructose and glucose for 14 days produced 10 and nine adventitious shoots respectively; and after 21 day incubation adventitious shoot count reached 19 in glucose supplemented medium. *S. racemosa* explants in the same experiment produced five shoots in maltose and four shoots in sorbitol supplemented medium after 14 day incubation; whereas after 21 day incubation, sucrose and maltose produced five shoots; fructose, glucose, and sorbitol produced four shoots. Regenerated plants were successfully acclimatized and Scanning Electron Microscopy of the leaf surface revealed differences in stomatal behavior and cuticle deposition between *in vitro* and acclimatized plants. The antioxidant assay conducted on both *Scutellaria* species showed considerable total polyphenol content, TEAC activity and flavonoid content in fresh and dried leaf samples attributing to their medicinal potential.

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Keywords

Antioxidant, 6-Benzylaminopurine, Flavonoid, *meta*-Topolin, Polyphenol, RIM, SIM

1. Introduction

Family Lamiaceae is often known for the plants with aromatic and herbal uses. Basil (*Ocimum* spp.), mint (*Mentha* spp.), rosemary (*Rosmarinus* spp.), sage (*Salvia* spp.), oregano (*Origanum* spp.), and lavender (*Lavandula* spp.) are some of the plants that are well known for their therapeutic and culinary properties. *Scutellaria* is a large genus represented by over 360 species that grow around the world from wet to xeric condition [1]. *S. baicalensis* Georgi is used in Traditional Chinese Medicine (TCM) and Japanese Kampo medicine for over 2000 years to treat bronchitis, hepatitis, diarrhea, and tumors [2].

Scutellaria barbata D. Don is native to Asia and commonly known as barbed skullcap. It is a perennial herb reaching 35 - 40 cm tall. The leaves are about three centimeters long. The purple-blue, lightly hairy flower is roughly a centimeter long. The plant grows in moist and wet habitat. *Scutellaria racemosa* Pers. is an herbaceous perennial with a sprawling habit, slender spreading rhizomes, and a stem height of 10 - 30 cm. *S. racemosa* is also known as South American Skullcap. The flower is tube shaped like the *S. barbata* but the color can vary from pale lavender to pink and white mottled, are 6 - 10 mm long. The plant is able to produce flowers and fruits throughout the year [3]. Kral [4] reported the appearance of *S. racemosa* in the southeast U.S. Ethnobotanical information suggests that the Cauca people of Columbia and Ecuador used certain ecotypes of *S. racemosa* in ceremonial or sedative preparations [5]. *S. barbata* has been tested in two clinical trials for the treatment of advanced and metastatic breast cancer with positive results [6] [7]. Laboratory studies using Barbat skullcap extracts have shown to induce apoptosis in prostate cancer, and hepatoma H22 cells [8]-[10]. Biomedical studies using animal model, exhibited improved antidepressant action of *S. racemosa* extract [5]. Recently, four new compounds were extracted and identified from *S. racemosa*, the triterpenoid lupeol and the flavonoids oroxylin A (5,7-dihydroxy-6-methoxyflavone), hispidulin (4',5,7-trihydroxy-6-methoxyflavone), and oroxyloside (oroxylin A 7-O-glucuronide). These compounds are involved in the selective inhibition of prolyl oligopeptidase [11].

The production of medicinal plants that are free from biotic contaminants like bacteria, fungus, and insects is important for the safety of consumers taking complementary and alternative medicine. The challenge for herb growers is to procure high quality seed and planting stock as it can be difficult to obtain and at times very expensive. Many of the medicinal herb seeds are collected by wild-crafting and the seeds are extremely variable in viability and germination rate. Batch to batch seed germination can be very slow and erratic leading to sporadic plantings in the field. Many medicinal herb seeds have specific and long stratification requirements. *In vitro* plant propagation techniques help to produce select clones of superior individual genotypes [12]. Successful micropropagation of *Scutellaria* has been reported for various species [12]-[20]. In these species mostly BAP and TDZ have been found to be the best cytokinin for shoot bud induction. Various types of cytokinins at varied concentrations, individually or in combination, have been tried in regeneration studies in many plants [21]-[23]. Regeneration studies on anticancer plant *Solanum viarum*, sugars (sucrose, glucose, fructose, and maltose) were incorporated to determine their effect on shoot induction [24]. In this study fructose (4% w/v) added to Murashige and Skoog medium produced the highest number of shoots [24] [25]. A reliable micropropagation protocol will help rapid multiplication of desirable species, conservation of rare and endangered plants, and scaling up herbal biomass production for commerce. The aim of this study is to find the optimal cytokinin, carbon source, and acclimation requirements to produce these two *Scutellaria* species *in vitro*. We also present Scanning Electron Microscopic (SEM) studies to record micromorphological information on leaves to assist acclimatization step. In literature, medicinal plant species have been shown to possess high antioxidant capacity [20] [26] [27]. We also present antioxidant capacity estimation of extracts obtained from fresh and dry leaves of *S. barbata* and *S. racemosa*.

2. Materials and Methods

2.1. Role of Cytokinins and NAA on Shoot Bud Induction

2.1.1. Media Preparation

Premixed Murashige and Skoog medium (MS 519, *Phyto*Technology Laboratories, KS, USA) was supple-

mented with 30 g·L^{-1}sucrose, 7 g·L^{-1} agar (pH 5.8) and was used for shoot induction medium (SIM). On the basis of previous studies on *Scutellaria* tissue culture conducted in our lab, shoot induction medium contained 5 µM cytokinin in combination with 0.1 µM NAA [13]-[17]. Plant growth regulators, 6-benzylaminopurine (BAP), 6-furfurylaminopurine (Kinetin), N-phenyl-N'-1, 2, 3-thiadiazol-5-ylurea (thidiazuron-TDZ), 1-naphthylacetic-acid (NAA) (Sigma Chemical Co., MO, USA), 6-(γ, γ-dimethylallylamino)purine (2iP), 6-(3-hydroxybenzyla mino)purine (*meta*-Topolin-*m*-Topolin) (*Phyto*Technology Laboratories, KS, USA), and 6-(4-Hydroxy-3-methylbut-2-enylamino)purine (Zeatin) (Caisson Laboratories, UT, USA) were added to MS basal medium. Each treatment had five replicates and 15 mL of medium was dispensed in each tube with control treatment without any plant growth regulators.

2.1.2. Explant Preparation and Culturing

Two month old *in vitro* mother stock plants of *S. barbata* and *S. racemosa* were established on MS basal medium. Mother stock was developed using shoot tip and nodal explants to harvest explants for all experiments. For the evaluation of the effect of various cytokinins on shoot bud induction, 10 mm ± 2 mm nodal explants without leaves were excised from healthy, vigorously growing shoots under sterile conditions. Cultures were maintained at 25˚C ± 2˚C with 16 h photoperiod provided by cool white fluorescent tubes with an approximate light intensity of 40 µmol m^{-2}·s^{-1}. All cultures for shoot bud induction, elongation, root induction, and acclimatization experiments were kept under these same conditions. Explants were transferred from each cytokinin treatment randomly to semi-solid MS basal elongation medium after 14 and 21 days of incubation in the SIM.

2.2. Optimized SIM Supplemented with Sugars and *in Vitro* Response

2.2.1. Media Preparation

For this study, Sucrose (34.2 g) (EMD Chemicals Inc., NJ, USA), D-Maltose (34.2 g), Fructose (18.0 g), D-Sorbitol (18.2 g) (*Phyto*Technology Laboratories, KS, USA), D-Glucose (18.0 g), D(+)-Mannose (18.0 g), *myo*-Inositol (18.0 g) (Sigma Chemical Co., MO, USA), and D-Mannitol (18.2 g) (DIFCO Lab., MI, USA) were added individually at 0.1 M concentration to further optimize shoot induction medium obtained through cytokinin experiment. MS medium was supplemented with 5 µM *m*-Topolin and 0.1 µM NAA, 7 g·L^{-1} of agar and pH was adjusted to 5.8 prior to autoclaving. The control had 0.1 M sucrose with no *m*-Topolin and NAA.

2.2.2. Elongation Medium

Five explants from each carbon source treatment were randomly picked and transferred into the elongation medium after 14 and 21 days of culturing. No plant growth regulators were added to elongation medium and the level of sucrose was reduced to 10 g·L^{-1}.

2.2.3. Rooting and Acclimatization

Root induction medium (RIM) was based on MS medium with vitamins, supplemented with 10 g·L^{-1} sucrose, 5 µM indole-3-butyric acid (IBA) (Sigma Chemical Co., MO, USA), 8 g·L^{-1} of agar and the pH was adjusted to 5.8 prior to autoclaving. Nodal explants with clump of shoots from elongation medium were transferred to the RIM for three weeks. A two Magenta™ box (GA-7, Magenta Corp., IL, USA) connected by an extender ring assembly was used for the rooting and acclimatization experiment (**Figure 3(C)**). A 1:1 mixture of perlite (Carolina Perlite Co. Inc., NC, USA) and growing medium (Metro-Mix® 360) (Sun Gro Horticulture Dist. Inc., MA, USA) moistened with liquid MS medium supplemented with 30 g·L^{-1} sucrose, 5 µM IBA, and Plant Preservative Mixture 5% (PPM™, Plant Cell Technology, DC, USA) was used as the substratum. The substratum mixture was placed in the bottom magenta box filling it half way and the closed unit was autoclaved.

After 21 days, rooted plantlets from RIM were removed under sterile conditions and any semi-solid medium on the roots was gently dislodged. Acclimatization of rooted shoots was carried out in the autoclaved substratum filled acclimatization boxes; 38 for *S. barbata* and 49 boxes for *S. racemosa*. After two weeks the boxes were cracked open slightly in the culture room and watered with tap water to start acclimation. Over the next two weeks the covers were opened further and watering continued. After a total of four weeks of acclimatization the plantlets were transferred to the greenhouse and planted in pots (Regal Standard Pot #STD0400, Park Seed Wholesale, Inc., SC, USA) with 1:1 mixture of perlite and growing medium moistened with tap water.

2.3. Scanning Electron Microscopy

Prior to visualizing plant material under scanning electron microscope (S 3400N, Hitachi High Technologies America Inc., CA, USA), samples were processed through fixation, dehydration, critical point drying, and sputter coating. In brief, primary fixation of the plant material was done in 2% Gluteraldehyde (Electron Microscopy Sciences, PA, USA) in Sorensen's Phosphate Buffer Saline (PBS) (pH 7.2) (Electron Microscopy Sciences, PA, USA) for one hour at 25˚C. After washing three times for 15 min each time in PBS buffer, secondary fixation was carried out using 1% Osmium tetroxide (Electron Microscopy Sciences, PA, USA) in PBS for one hour at 25˚C. Plant tissue was again washed three times for 15 min in distilled water at the end of one hour of secondary fixation. The dehydration process followed ascending series of ethanol from 25%, 50%, 75%, 85%, 95% to 100%. The final dehydration step was completed by placing in 100% ethanol for three changes of 15 min in each. After completion of dehydration, critical point drying (CPD) was followed (EMS850, Electron Microscopy Sciences, PA, USA). Sample fragments were mounted with two-sided adhesive carbon disc to specimen stubs, and sputter coated using a Denton Vacuum Desk V Cold Sputter/EtchUnit (Denton Vacuum LLC, NJ, USA). The gold palladium coating was at 50 Å. Coating was carried out for 60 s under a vacuum pressure of 0.05 torr using 30 mA current. Digital images were collected at various magnifications, running beam at 10 kV and keeping specimen stub at 4.4 mm distance. All scanning works were conducted at the Agricultural Research Station, Fort Valley State University, Fort Valley, Georgia, USA.

2.4. Determination of Antioxidant Capacity

2.4.1. Leaf Collection

S. barbata and *S. racemosa* plants grown in Fort Valley State University's greenhouse, Fort Valley, Georgia were used for this study. Rosemary (*Rosemarinus officinalis*), used as a standard, was collected locally. *Scutellaria* and rosemary leaves were harvested fresh and after gentle wash, excess moisture was dabbed off and weighed. Total weight of each of the fresh leaf samples was two grams; one was extracted fresh and other one was extracted after drying. Drying of the leaves was conducted at room temperature (25˚C ± 2˚C) for seven days. Dried samples were weighed and processed for extraction.

2.4.2. Leaf Extracts Preparation

Two grams of fresh leaf samples of each species were homogenized with liquid nitrogen in a 50 mL capacity chilled mortar and pestle. Homogenized leaf powder was transferred to 125 mL Erlenmeyer flask, in which 50 mL of HPLC grade Methanol (Burdick and Jackson, NJ, USA) was added for extraction of tissues overnight (18 h at 28.5˚C), under darkness using an orbital shaker at 200 rpm, with constant agitation (Benchmark Mini Incu-shaker, NJ, USA). After 18 h, suspension was placed in 50 mL falcon tubes (BD, NJ, USA), centrifuged at 4000 rpm (5810 R, Eppendorf, NY, USA) at room temperature for 40 min. The supernatant was collected in a new falcon tube and the remaining pellet was extracted as outlined above one more time, for one hour, in 25 mL of methanol. After the second extraction, two extracts were combined and the pellets were discarded. Extracts were filtered through a double layer of Whatman filter No. 2 (GE Healthcare Life Sciences, PA, USA) and stored at 4 ˚C in dark, for downstream process.

2.4.3. Antioxidant Capacity Studies

Total Polyphenol (TPP) content measurement was determined by the Folin-Ciocalteu reagent method Lowry *et al.* [28] modified by Yi and Wetzstein [29] for Lamiaceae family and further optimized for *Scutellaria* species by Vaidya [20]. Similarly antioxidant capacity measurement [TROLOX Equivalent Antioxidant Capacity (TEAC) Assay] was conducted as described by Re *et al.* [30] with modification from Yi and Wetzstein [29] for Lamiaceae family and again optimized for *Scutellaria* species by Vaidya [20]. Estimation of total flavonoid content was done by Aluminum chloride colorimetric method developed by Chang *et al.* [31] with modification for *Scutellaria* species by Vaidya [20].

2.5. Data Collection and Statistical Analysis

Observations on the number of adventitious buds induced in response to cytokinins in treatments were recorded after 63 days (nine weeks). Observations on the number of adventitious shoot buds induced on optimized SIM, supplemented with various sugars, were conducted after 49 days (seven weeks). The General Linear Model was

used to determine if there were any significant differences among the cytokinins in treatments and also for the carbon source treatments for shoot count. Where there was statistical significance ($p < 0.05$), the mean values were further separated using Student-Newman Keuls test. For antioxidant studies analysis of variance (ANOVA) single factor was performed to compare the means for significant difference between the treatments at $p < 0.05$ level. Where there was statistical difference Tukey's HSD test was performed.

3. Results

3.1. Role of Cytokinins and NAA on Shoot Bud Induction

Nodal explants take 7-10 days to get established in the SIM and axillary bud break is evident by the end of second week in both species. In case of *S. barbata*, the highest shoot count mean of four was seen in *meta*-Topolin treatment for 14 and 21 day incubation periods both (**Figure 1**). After 14 days in SIM, BAP and Kinetin both induced three shoots each on average, whereas after 21 days Zeatin treated explants resulted in three shoots per explant. The shoot count means for *S. racemosa* at the 14 and 21 day incubation periods also showed *meta*-Topolin as the most effective cytokinin with four and five shoots, respectively (**Figure 1**). Next to *meta*-Topolin, BAP treated explants recorded three shoots after 14 days of incubation and after 21 days of incubation shoot number reached four. Kinetin, TDZ, and 2iP in both species on an average registered two shoots at both time points which were similar to control without any plant growth regulator.

3.1.1. Optimized SIM Supplemented with Sugars and *in Vitro* Response

Control treatment with 0.1 M sucrose but without plant growth regulators produced two shoots per explant on an average for both species after 14 and 21 day incubation. The shoot count means for *S. barbata* nodal explants after 14 day incubation period resulted in 10 and nine shoots in fructose and glucose supplemented SIM. Maltose added medium followed with seven shoots per explant on average. After 21 day incubation period, shoot number in glucose supplemented SIM was recorded at 19 whereas fructose incorporated SIM registered 13 shoots (**Figure 2**). *S. racemosa* nodal explants in maltose supplemented SIM induced five shoots after 14 day incubation on average, followed by four shoots in response to sorbitol added medium. After 21 day incubation, the average shoot number recorded was five for maltose and sucrose and four in response to sorbitol supplemented SIM (**Figure 2**). After 21 days, fructose and glucose supplemented treatments resulted in four shoots each per explant. Both *Scutellaria* species were unable to metabolize mannose resulting in the death of nodal explants in the SIM.

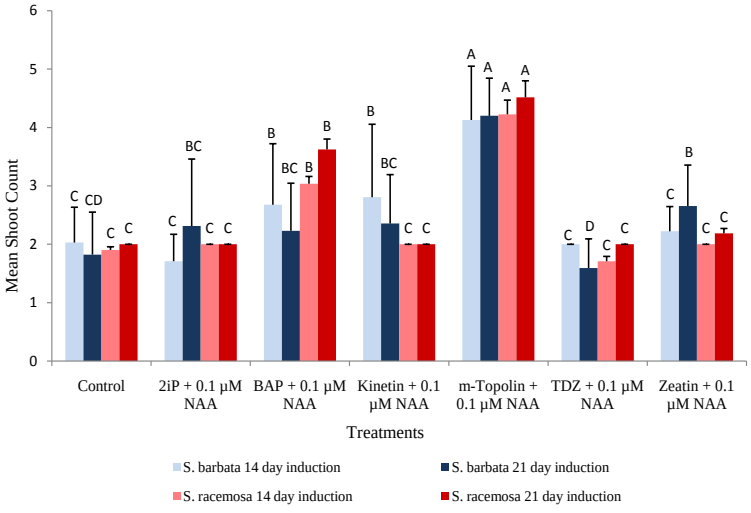

Figure 1. Effect of various cytokinin treatments on adventitious shoot bud induction in *S. barbata* and *S. racemosa* using nodal explants. Treatments with the same letters are not significantly different ($p < 0.05$) when compared at each induction period for each species separately. The values represent means of five replicates.

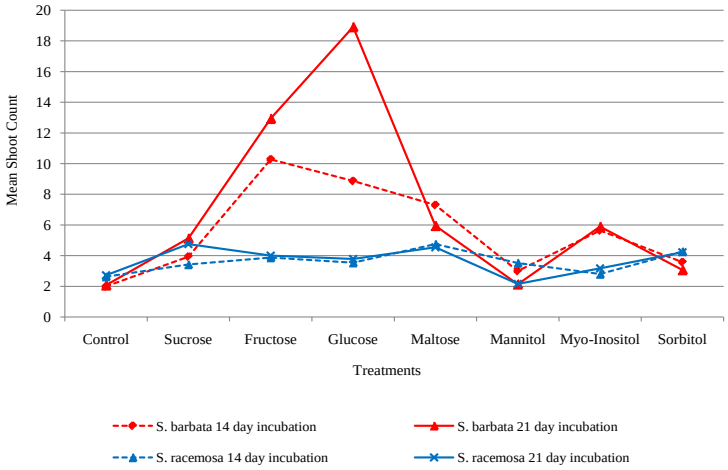

Figure 2. Effect of carbon source (type of sugar) on adventitious shoot bud induction in *S. barbata* and *S. racemosa*. Nodal explants were cultured in optimized SIM (MS + 5 µM *m*-Topolin + 0.1 µM NAA). Sugars were supplemented in the medium at 0.1 M. The values represent means of five replicates.

3.1.2. Rooting

All shoots from both species transferred to MS + IBA medium (RIM) for rooting survived and root induction was evident after three weeks. At this point plants were removed from RIM and were transferred to magenta box assembly for further root development, growth, and acclimatization. After four weeks in magenta box assembly, rooting for *S. barbata* and *S. racemosa* was recorded at 98% and 85%, respectively. The final step for acclimatization was to transfer plants from magenta boxes to the greenhouse. Plants in the greenhouse were watered two times a day and had shade cloth allowing 50% sunlight. The survivability of acclimatized plants of *S. barbata* was 80% and for *S. racemosa* it was 95%.

3.1.3. Scanning Electron Microscopy of Leaf Surface

Leaves from greenhouse, three weeks cultured in SIM, and acclimatized for three weeks were processed for scanning electron microscopy of abaxial surface for both species (**Figure 4(A)** and **Figure 4(F)**). Stomata are well distributed on the abaxial surface in both species. There was striking difference in the stomatal behavior as leaves from three weeks old explants in SIM exhibited open stomata and the response was more pronounced in case of *S. barbata* (**Figure 4(B)** and **Figure 4(E)**). After three weeks of acclimatization, stomatal function was regained.

3.1.4. Antioxidant Capacity

The total polyphenol content (TPP) for fresh leaf extracts of *S. barbata* was 69.7 mg/g gallic acid equivalent (GAE) and *S. racemosa* was 78.9 mg/g GAE compared to the internal standard of *Rosemarinus officinalis* (Rosemary) which was 175.5 mg/g GAE. The TEAC values for *S. barbata* was 716.7 µmol/g, for *S. racemosa* it was 637.8 µmol/g and *R. officinalis* had the highest TEAC value of 1344.1 µmol/g. The dry extracts of these two *Scutellaria* species exhibited lower values in TPP; *S. barbata* dry leaf extract exhibited 56.5 mg/g GAE *while S. racemosa* exhibited 51.5 mg/g GAE but the internal standard of *R. officinalis* fresh leaf extracts registered 325.3 mg/g GAE. The TEAC value for *S. barbata* was 741.1 µmol/g, *S. racemosa* was 410.8 µmol/g and for *R. officinalis* it was 2092.1 µmol/g respectively. The data for both fresh and dry extracts show that TPP values increased as the TEAC values increased and as TPP values decreased, TEAC values also decreased, following a pattern suggesting a strong correlation between TPP and TEAC assays (**Table 1**). The *R. officinalis* in both fresh and dry extracts have higher TPP and TEAC values suggesting its high antioxidant activities.

For estimation of total flavonoid content using Aluminum chloride method, fresh extract of each species tested exhibited higher values; *S. barbata* had 143.0 µg/mL, *S. racemosa* had 89.6 µg/mL and *R. officinalis* had 132.8 µg/mL. In the dry extracts result, *S. racemosa* had lowest value with 61.2 µg/mL, followed by *S. barbata* with 99.1 µg/mL and *R. officinalis* with 131.7 µg/mL. The fresh and dry leaf extracts of *R. officinalis* did not differ in flavonoid content.

Table 1. Total polyphenol, TROLOX Equivalent Antioxidant content, content estimation of fresh and dry leaf extracts of *S. barbata*, *S. racemosa*, and *R. officinalis*. The values represent means of three replicates. The analysis of variance (ANOVA) single factor for fresh and dry extracts were performed to compare the means with significant difference between treatments at $p < 0.05$ level.

Species	TPP (mg/g GAE)		TEAC (µmol/g)		Flavonoid content (µg/mL)	
	Fresh	Dry	Fresh	Dry	Fresh	Dry
S. barbata	69.7 ± 0.1	56.5 ± 0.5	716.7 ± 12.0	741.1 ± 37.5	143.0 ± 15.8	99.1 ± 2.5
S. racemosa	78.9 ± 0.2	51.5 ± 0.1	637.8 ± 57.4	410.8 ± 31.0	89.6 ± 6.7	61.2 ± 3.7
R. officinalis	175.5 ± 0.4	325.3 ± 1.1	1344.1 ± 3.9	2092.1 ± 64.8	132.8 ± 20.1	131.7 ± 14.6

4. Discussion

Earlier studies on the micropropagation of *Scutellaria* spp. have shown that cytokinins used above 5 µM concentration either suppressed shoot bud development or induced hyperhydricity [13] [15]-[17]. This is true for liquid and semi-solid cultures both, though the incidence was lower in agar based medium and species dependent. Higher TDZ concentrations caused hyperhydricity in *S. altissima* cultures [32]. In the current study, *meta*-Topolin emerged as the best cytokinin for both *Scutellaria* species (**Figure 3(A)** and **Figure 3(F)**). Adelberg and Naylor-Adelberg [33] tested benzyladenine and *meta*-Topolin on the multiplication and rooting of *Aloe barbadensis* and found that *meta*-Topolin at 10 µM and benzyladenine at 3.2 µM had the best shoot regeneration. Sea oats (*Uniola paniculata* L.) have genotypes that are both easy and difficult-to-acclimatize, were cultured on 2.2 µM BAP and *meta*-Topolin supplemented medium. It was seen that ex vitro acclimatization of the difficult-to-acclimatize genotype was much better with *meta*-Topolin than with BAP [34]. Keeping this in mind, various cytokinins at 5 µM level were tested and another factor (various carbon sources) was experimented in the present study to control hyperhydricity and enhance shoot bud induction. It was found that *meta*-Topolin exhibited highest shoot bud induction in *S. barbata* and *S. racemosa* both, and shoot number could be further enhanced by addition of sugar; glucose and fructose in case of *S. barbata* and sucrose and maltose in case of *S. racemosa* (**Figure 2**, **Figure 3(B)** and **Figure 3(G)**). Shoot bud induction studies on *Citrus* epicotyls revealed that cytokinins BAP and *meta*-Topolin were equally responsive but at different concentrations [35]. Genotypic differences have been shown to play an important role in preferred uptake of carbon source supplemented in the shoot induction medium in other plants too [36]-[38]. In case of MM.106 apple rootstock, fructose, sucrose, glucose, sorbitol, and maltose along with 4.43 µM BAP and 0.4 µM IBA was tested on *in vitro* shoot induction; sorbitol at 90 mM being the most effective sugar [36]. The effect of the proper carbon source and concentration also determines the health and viability of the *in vitro* plants. Sujana and Naidu [37] tested sucrose, maltose, glucose, and fructose at 1%, 2%, 3%, 4%, 5%, and 6% on *Mentha piperita* (L.) and found fructose at 4% was the best for plant regeneration. In a detailed study on Japanese Pear "Hosui", testing sugars sorbitol, sucrose, fructose, glucose, maltose, lactose, and mannitol at 30, 60, and 120 mM concentrations, sorbitol at 60 mM was found as the best sugar for shoot proliferation [38]. Acclimatization protocols developed for *S. barbata* and *S. racemosa* in this study registered high rate of plant survival in the greenhouse and SEM studies clearly indicate the progress with the regulation of stomatal functioning and cuticle deposition (**Figure 4**). In a similar SEM study on *in vitro* versus field grown *Celastrus paniculatus* Willd., the *in vitro* plants exhibited higher number of fully opened stomata and reduced epicuticular wax deposition [39]. This was also seen in our study where stomata on *in vitro* leaves were fully opened all the time. However, after acclimatization the stomata could be seen in various stages from closed to open state (**Figure 4**).

Drying of leaves for extract preparation produced variable results (**Table 1**). Drying resulted in considerable increase of total flavonoids and TEAC activity in case of *S. barbata* whereas *S. racemosa* exhibited opposite trend. Similar examples have been reported in peppermint (*Mentha x piperita* L.), lemon balm (*Melissa officinalis* L.) and oregano (*Origanum vulgare* L.) where drying resulted in considerable loss in the quantity of L-Ascorbic acid and of carotenoids, hence drastic reduction in antioxidant activity [40]. Jambor and Czosnowska in 2002 [41] suggested that this reduction in antioxidant capacity may arise due to changes in enzymatic process during drying which could change the phytochemical composition in the plant. Research on the comparison of common culinary and medicinal herbs that were grown in the greenhouse and field conditions suggested higher

Figure 3. Micropropagation of *Scutellaria barbata* (A)-(E) and *S. racemosa* (F)-(I). (A)-(D) Various steps in the micropropagation of *S. barbata*. (A) and (F) Regeneration in nodal explant of *S. barbata* and *S. racemosa* after three weeks each in shoot induction (5 µM *meta*-Topolin and 0.1 µM NAA) and elongation medium (MS basal); (B) and (G) Nodal explants exhibiting regeneration after three weeks in shoot induction medium with various carbon sources and four weeks in elongation medium, control and 0.1M glucose cultures in B and control and 0.1 M Sucrose treated cultures in (G), (C) Microshoots from *S. barbata* and *S. racemosa* were transferred to root induction medium (RIM) for three weeks and then to magenta box assembly with 1:1 perlite and growing substrate moistened with liquid MS medium with 5µM IBA and 5% PPM; (D) and (H) After four weeks in magenta boxes for rooting/acclimation plants were healthy and well rooted; left to right: control, fructose, glucose, maltose, sucrose, *myo*-Inositol, mannitol, and sorbitol. (E) and (I). Acclimated plants from (D) and (H) were transferred to greenhouse for further growth, flowering, and seed set.

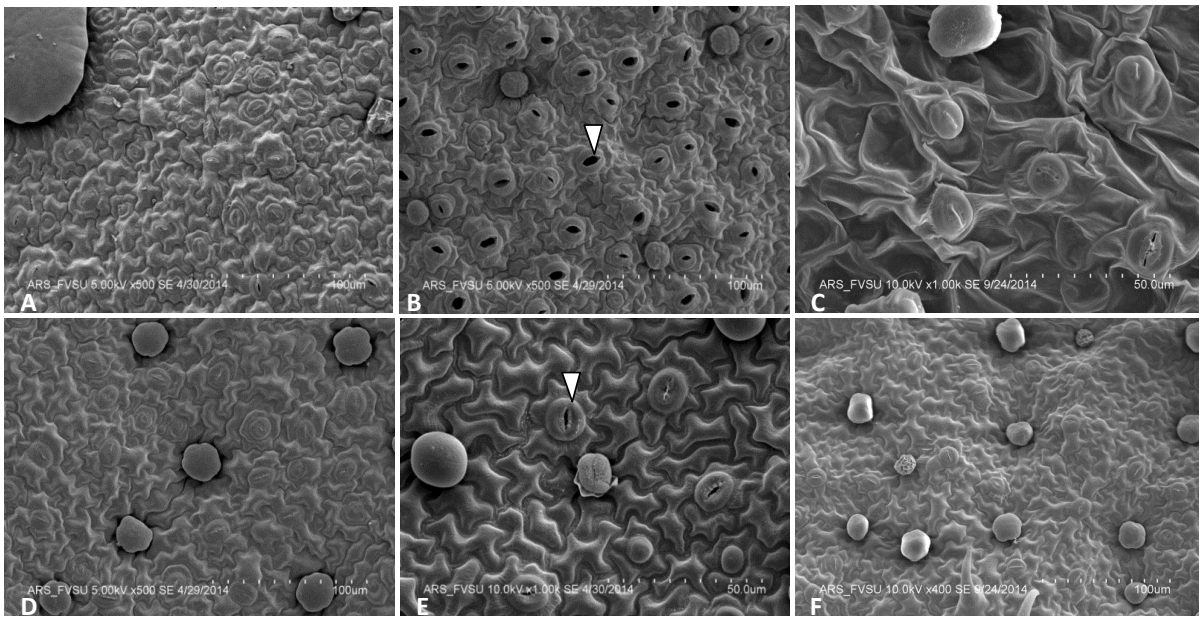

Figure 4. Scanning electron microscopy of *S. barbata* (A)-(C) and *S. racemosa* leaves (D)-(F). (A) and (D) Fresh leaves from donor plants maintained in the greenhouse showing abaxial surface. (B) and (E) Abaxial surface from three week old cultures in shoot induction medium showing majority of stomata open, and (C) and (F) Abaxial surface of leaves after three weeks of acclimatization with stomatal functioning regained and cuticle layer restored.

TPP, TEAC, and flavonoid contents in greenhouse grown plants, indicating the role of environmental factors on the accumulation of bioactive compounds [29]. It has been suggested that not only the level of antioxidants but also a synergy occurring among them and the other plant constituents might influence the differences in the antioxidant capacity of plant extracts [42]. The relationship between *Scutellaria* extract containing flavonoids and

their role in anti-tumor properties via Akt/GSK-3 signaling pathway has been established [43] [44].

5. Conclusion

A major thrust in our research is to reduce hyperhydricity condition in tissue cultured plants as this physiological aberration reduces productivity [17]. This study suggests that *S. barbata* and *S. racemosa* can be successfully cultured *in vitro* by using lower concentrations of cytokinin for a brief period (14 - 21 days) and optimizing type of sugar in the same shoot induction medium to further enhance the number of shoots. It would be interesting to study the incidence of hyperhydricity in explants incubated for a shorter incubation period (7 - 10 days) with higher than 5 µM concentration of cytokinins. Further, successful rooting and acclimatization protocols were developed resulting in minimal mortality. All these studies will help understand medicinal plant physiology for scaling up biomass production to assist commercial production. Scanning electron microscopic studies highlight the adverse effect of *in vitro* condition on leaf morphology and development providing insight into minimum duration of hardening required. A suitable strategy for acclimatization of micropropagated plants is needed which could correct stomatal abnormalities and initiate synthesis of cuticle on the leaf surface. Further, both species exhibited high antioxidant capacity which could be a reason behind their traditional medicinal use and recent clinical studies.

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