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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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, PhytoTechnology 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 \(\mu\)M cytokinin in combination with 0.1 \(\mu\)M NAA [13]-[17]. Plant growth regulators, 6-benzylaminopurine (BAP), 6-furfurylaminopurine (Kinetin), N-phenyl-N’-1, 2, 3-thiadiazol-5-y lurea (thidiazuron-TDZ), 1-naphthylacetic acid (NAA) (Sigma Chemical Co., MO, USA), 6-(\(\gamma\), \(\gamma\)-dimethylallylamino)purine (2iP), 6-(3-hydroxybenzylamino)purine (meta-Topolin-m-Topolin) (*Phyto Technology Laboratories, KS, USA), and 6-(4-Hydroxy-3-methylbut-2-ethylamino)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 \(\mu\)mol 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 \(\mu\)M *m*-Topolin and 0.1 \(\mu\)M NAA, 7 g L\(^{-1}\) 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 \(\mu\)M 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 \(\mu\)M 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% Glutaraldehyde (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/Etch Unit (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 (*Rosmarinus 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 Incubator, 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.
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.
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

| 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 |
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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References

[1] Paton, A. (1990) A Global Taxonomic Investigation of *Scutellaria* (Labiatae). *Kew Bulletin*, 45, 399-450. [http://dx.doi.org/10.2307/4110512](http://dx.doi.org/10.2307/4110512)

[2] Zobayed, S.M.A., Murch, S.J., Rupasinghe, H.P.V., de Boer, J.G., Glickman, B.W. and Saxena, P.K. (2004) Optimized System for Biomass Production, Chemical Characterization and Evaluation of Chemo-Preventive Properties of *Scutellaria baicalensis* Georgi. *Plant Science*, 167, 439-446. [http://dx.doi.org/10.1016/j.plantsci.2004.04.022](http://dx.doi.org/10.1016/j.plantsci.2004.04.022)

[3] Krings, A. and Neal, J. (2001) South American Skullcap (*Scutellaria racemosa*: Lamiaceae) in the Southeastern United States. *SIDA*, 19, 1171-1179.

[4] Kral, R. (1973) Some Notes on the Flora of the Southern States, Particularly Alabama and Tennessee. *Rhodora*, 75, 366-410.

[5] Bianchi, A. (2006) Antidepressant Activity of Two Species of Colombian *Scutellariae*. *Proceeding from 6th Conference AMIAR*, Turin, 8 April 2006, 45-46.

[6] Perez, A.T., Arun, B., Tripathy, D., Tagliaferri, M.A., Shaw, H.S., Kimmick, G.G., Cohen, I., Shitivelman, E., Caygill, K.A., Grady, D., Schachtman, M. and Shapiro, C.L. (2010) A Phase 1B Dose Escalation Trial of *Scutellaria barbata* (BZL101) for Patients with Metastatic Breast Cancer. *Breast Cancer Research and Treatment*, 120, 111-118. [http://dx.doi.org/10.1007/s10549-009-0678-5](http://dx.doi.org/10.1007/s10549-009-0678-5)

[7] Rugo, H., Shitivelman, E., Perez, A., Vogel, C., Franco, S., Chiu, E.T., Melisko, M., Tagliaferri, M., Cohen, I., Shoemaker, M., Tran, Z. and Tripathy, D. (2007) Phase I Trial and Antitumor Effects of BZL101 for Patients with Advanced Breast Cancer. *Breast Cancer Research and Treatment*, 105, 17-28. [http://dx.doi.org/10.1007/s10549-006-9430-6](http://dx.doi.org/10.1007/s10549-006-9430-6)

[8] Dai, Z.J., Wang, X.J., Li, Z.F., Ji, Z.Z., Ren, H.T., Tang, W., Liu, X.X., Kang, H.F., Guan, H.T. and Song, L.Q. (2008) *Scutellaria barbata* Extract Induces Apoptosis of Hepatoma H22 Cells Via the Mitochondrial Pathway Involving Caspase-3. *World Journal of Gastroenterology*, 14, 7321-7328. [http://dx.doi.org/10.3748/wjg.14.7321](http://dx.doi.org/10.3748/wjg.14.7321)

[9] Marconett, C.N., Morgenstern, T.J., San Roman, A.K., Sundar, S.N., Singhal, A.K. and Firestone, G.L. (2010) BZL101, a Phytochemical Extract From the *Scutellaria barbata* Plant, Disrupts Proliferation of Human Breast and Prostate Cancer Cells through Distinct Mechanisms Dependent on the Cancer Cell Phenotype. *Cancer Biology & Therapy*, 10, 397-405. [http://dx.doi.org/10.4161/cbt.10.4.12424](http://dx.doi.org/10.4161/cbt.10.4.12424)

[10] Wong, B.Y., Nguyen, D.L., Lin, T., Wong, H.H., Cavalcante, A., Greenberg, N.M., Hausted, R.P. and Zheng, J. (2009) Chinese Medicinal Herb *Scutellaria barbata* Modulates Apoptosis and Cell Survival in Murine and Human Prostate...
Cancer Cells and Tumor Development in TRAMP Mice. European Journal of Cancer Prevention, 18, 331-341. 
[11] Marques, M.R., Stüker, C., Kichik, N., Tarragó, T., Giralt, E., Morel, A.F. and Dalcol, I.I. (2010) Flavonoids with Prolyl oligopeptidase Inhibitory Activity Isolated from Scutellaria racemosa Pers. Fitoterapia, 81, 552-556.
[12] Li, H., Murch, S.J. and Saxena, P.K. (2000) Thidiazuron-Induced de Novo Shoot Organogenesis on Seedlings, Etiolated Hypocotyls and Stem Segments of Huang-Qin. Plant Cell, Tissue and Organ Culture, 62, 169-173. 
[13] Joshee, N., Mentreddy, S.R. and Yadav, A.K. (2007) Mycorrhizal Fungi and Growth and Development of Micropropagated Scutellaria integrifolia Plants. Industrial Crops and Products, 25, 169-177. 
[14] Joshee, N., Tascan, A., Medina-Bolivar, F., Parajuli, P., Rimando, A.M., Shannon, D.A. and Adelberg, J.W. (2012) Scutellaria: Biotechnology, Phytochemistry and Its Potential as a Commercial Medicinal Crop. In: Chandra, S., Lata, H. and Varma, A., Eds., Biotechnology for Medicinal Plants: Micropropagation and Improvement, Springer-Verlag, Heidelberg, 69-99.
[15] Tascan, A. (2007) Liquid Culture Systems for Scutellaria Species in Vitro. M.S. Thesis, Clemson University, Clemson.
[16] Tascan, A., Adelberg, J.W., Joshee, N., Yadav, A.K. and Tascan, M. (2007) Liquid Culture System for Scutellaria Species. Acta Horticulturae, 756, 163-170.
[17] Tascan, A., Adelberg, J., Tascan, M., rimando, A., Joshee, N. and Yadav, A.K. (2010) Hyperhydricity and Flavonoid Content of Scutellaria Species in Vitro on Polyester-Supported Liquid Culture Systems. HortScience, 45, 1723-1728.
[18] Sinha, S., Vaidya, B., Pochrel, S. and Joshee, N. (1999) In Vitro Micropropagation and Callus Induction in Scutellaria discolor Covl.—A Medically Important Plant of Nepal. Indian Journal Plant Genetic Resources, 12, 219-223.
[19] Stojakowska, A., Malarz, J. and Kohlumuenzer, S. (1999) Micropropagation of Scutellaria baicalensis Georg. Acta Societatis Botanicorum Poloniae, 68, 103-107.
[20] Vaidya, B. (2013) AntioxidantPotential, Conservation, and Reproductive Biology of Medicinal Scutellaria. M.S. Dissertation, Fort Valley State University, Fort Valley.
[21] Aremu, A.O., Bairu, M.W., Doležal, K., Finnie, J.F. and Van Staden, J. (2012) Topolins: A Panacea to Plant Tissue Culture Challenges? Plant Cell, Tissue and Organ Culture, 108, 1-16. http://dx.doi.org/10.1007/s11240-011-0007-7
[22] Cole, I.B., Saxena, P.K. and Murch, S.J. (2007) Medicinal Biotechnology in the Genus Scutellaria. In Vitro Cellular & Developmental Biology-Plant, 43, 318-327. http://dx.doi.org/10.1007/s11627-007-9055-4
[23] Guo, B., Abbasi, B.H., Xu, L.L. and Wei, Y.H. (2011) Thidiazuron: A Multi-Dimensional Plant Growth Regulator. African Journal of Biotechnology, 10, 8984-9000.
[24] Mahadev, M.D.N., Panathula, C.S. and Naidu, C.V. (2014) Impact of Different Carbohydrates and Their Concentration on in Vitro Regeneration of Solanum viarum (Dunal)—An Important Anticancer Medicinal Plant. American Journal of Plant Sciences, 5, 200-204. http://dx.doi.org/10.4236/ajps.2014.51026
[25] Murashiget, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum, 15, 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb00852.x
[26] Sengul, M., Yildiz, H., Gungor, N., Cetin, B., Esen, Z. and Ercisli, S. (2009) Total Phenolic Content, Antioxidant and Antimicrobial Activities of Some Medicinal Plants. Pakistan Journal of Pharmaceutical Science, 22, 102-106.
[27] Vaidya, B.N., Brearley, T.A. and Joshee, N. (2013) Antioxidant Capacity of Fresh and Dry Leaf Extracts of Sixteen Scutellaria Species. Journal of Medicinally Active Plants, 2, 42-49.
[28] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent. The Journal of Biological Chemistry, 193, 265-275.
[29] Yi, W.G. and Wetzstein, H. (2010) Biochemical, Biological and Histological Evaluation of Some Culinary and Medicinal Herbs Grown Under Greenhouse and Field Conditions. Journal of the Science of Food and Agriculture, 90, 1063-1070.
[30] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999) Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. Free Radical Biology and Medicine, 26, 1231-1237. 
[31] Chang, C.C., Yang, M.H., Wen, H.M. and Chern, J.C. (2002) Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods. Journal of Food and Drug Analysis, 10, 178-182.
[32] Grzegorczyk, I. and Wysokińska, H. (2009) Micropropagation of Scutellaria altissima L. Proceedings of 12th National Conference “In Vitro Culture”, Poznań, 9-11 September 2009, 42.
[33] Adelberg, J., and Naylor-Adelberg, J. (2012) Effects of Cytokinin on Multiplication and Rooting of Aloe barbadensis
During Micropropagation on Agar and Liquid Medium. *Journal of Medicinally Active Plants*, 1, 1-5.

[34] Valero-Aracama, C., Kane, M.E., Wilson, S.B. and Philman, N.L. (2010) Substitution of Benzyladenine with *Meta*-Topolin during Shoot Multiplication Increases Acclimatization of Difficult and Easy-to-Acclimatize Sea Oats (*Uniola paniculata* L.) Genotypes. *Plant Growth Regulation*, 60, 43-49. http://dx.doi.org/10.1007/s10725-009-9417-5

[35] Niedz, R.P. and Evens, T.J. (2010) The Effects of Benzyladenine and *Meta*-Topolin on *in Vitro* Shoot Regeneration of a Citrus Citranadarin Rootstock. *Research Journal of Agriculture and Biological Sciences*, 6, 45-53.

[36] Bahmani, R., Gholami, M., Abdollahi, H. and Omid, K. (2009) The Effect of Carbon Source and Concentration on *in Vitro* Shoot Proliferation of MM.106 Apple Rootstock. *Fruit, Vegetable and Cereal Science and Biotechnology*, 3, 35-37.

[37] Sujana, P. and Naidu, C.V. (2011) Impact of Different Carbohydrates on High Frequency Plant Regeneration from Axillary Buds of *Mentha piperita* (L.)—An Important Multipurpose Medicinal Plant. *Journal of Phytology*, 3, 14-18.

[38] Kadota, M. and Niimi, Y. (2004) Influences of Carbon Sources and Their Concentrations on Shoot Proliferation and Rooting of “Hosui” Japanese Pear. *HortScience*, 39, 1681-1683.

[39] Rao, M.S., Suthar, D. and Purohit, S.D. (2014) Scanning Electron Microscopic Study Reveals Stomatal Malfunctioning in *in Vitro* Grown *Calastarus paniculatus* Willd. *International Journal of Advanced Life Sciences and Engineering*, 1, 44-50.

[40] Capecka, E., Mareczek, A. and Leja, M. (2005) Antioxidant Activity of Fresh and Dry Herbs of Some *Lamiaceae* Species. *Food Chemistry*, 93, 223-226. http://dx.doi.org/10.1016/j.foodchem.2004.09.020

[41] Jambor, J. and Czosnowska, E. (2002) Herbal Medicines from Fresh Plants. *Postępy Fitoterapii*, 8, 2-5.

[42] Zheng, W. and Wang, S.Y. (2001) Antioxidant Activity and Phenolic Compounds in Selected Herbs. *Journal of Agricultural and Food Chemistry*, 49, 5165-5170. http://dx.doi.org/10.1021/jf010697n

[43] Patel, P.S., Joshee, N., Rimando, A.M. and Parajuli, P. (2013) Anti-Cancer Scopes and Associated Mechanisms of *Scutellaria* Extract and Flavonoid Wogonin. *Current Cancer Therapy Reviews*, 9, 34-42.

[44] Parajuli, P., Joshee, N., Chinni, S.R., Rimando, A.M., Mittal, S., Sethi, S. and Yadav, A.K. (2011) Delayed Growth of Glioma by *Scutellaria* Flavonoids Involve Inhibition of Akt, GSK-3 and NF-κB Signaling. *Journal of Neuro-Oncology*, 101, 15-24. http://dx.doi.org/10.1007/s11060-010-0221-x
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