Callus Induction and Establishing Cell Suspension Culture of *Agastache* J.Clayton Ex Gronov

Oksana B Polivanova[1], *Mikhail Yu* Cherednichenko[1*], and *Daria A* Khlebnikova[1]

[1]Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Moscow, Russia

Abstract. The aim of this study was to initiate calli formation and cell suspension cultures from some species of *Agastache* genus. These plants could be useful for a production of bioactive secondary metabolites *in vitro*. For the initiation of callogenesis, two explant types were tested: leaf and stem explants from 40–60 days old *in vitro* seedlings. Percentage of callus formation was used as criterion to evaluate the efficiency of callus induction. Leaf- and stem-derived friable calli of *A. foeniculum* and *A. urticifolia* cultivated on MS medium supplemented with 0.5 mg/L 2.4-dichlorophenoxyacetic acid and 0.1 mg/L kinetin were selected for the cell suspension cultures establishing. The cell suspension cultures of *A. foeniculum* characterized by growth indexes of 1.08 and 8.57 for MS and B5 media respectively. For *A. urticifolia* suspension cultures growth indexes were 3.01 for MS medium and 1.29 for B5 medium. The period of culturing was 28 days. Viability of cell suspension cultures varied 50–100 during the period of culturing. According to the growth characteristics for establishing *A. foeniculum* suspension culture is better to use MS medium, and for *A. urticifolia* – B5.

1 Introduction

Agastache is a genus of *Lamiaceae* encompassing more than 20 species of perennial aromatic and medicinal plants native to North America (except one species, *A. rugosa*, native to Central Asia) and widely using in traditional medicine for the treatment of nausea, vomiting, bacterial infections, diarrhea, cholera and anxiety [1]. Some of *Agastache* species are applied as sources of essential oils, herbal drugs and spices as well as ornamental and nectariferous plants [2].

Agastache species contain valuable secondary metabolites such as phenylpropanoids and terpenoids. The first group includes flavonoids, free phenolic acids, together with caffeic acid derivatives, and depsids, such as lignans. Terpenoids of A*gastache* contain non-volatile and volatile components of essential oils [3]. Essential oils of *Agastache* have antibacterial, antifungal, cytotoxic, nematicidal activity and insecticidal properties [4]. The main components of *Agastache* essential oils are estragole, limonene, anisaldehyde, methyleugenol, thymol, pulegone [5-6]. The essential oil content depends on different

* Corresponding author: cherednichenko@rgau-msha.ru

factors such as a species, plant organ, harvesting time, environmental conditions and cultivation methods [3]. Estragole (syn.: methyl chavicol) is usually the most abundant compound of *A. rugosa* and A. *foeniculum* essential oils (over 98%) [7]. The caffeic acid derivatives such as rosmarinic acid (RA), ester of caffeic acid and 3.4-dihydroxyphenyllactic acid, and lithospermic acid B, a dimer of RA, are noted to possess some medical properties [8]. It has been found that RA has antioxidant properties associated with membranes stabilization. RA is the obstacle of radical propagation that protects membranes against oxidative damage [9]. RA also is a promising neuroprotective compound. It has a cognitive-enhancing effect and can inhibit aggregation of amyloid-β, which makes it potential therapeutic substance for Alzheimer's disease treatment [10]. RA and its derivatives are comparatively easy accumulate in cultured plant cells [11]. Suspension cultures and Hairy root cultures of *Agastache* were also studied as RA producers [12]. *Agastache* genus is a source of new chemical compounds with unidentified biological activity. Several new diterpenoids from roots of *A. rugosa*, for example, agastaquinone and other oxidized abietanoids, such as agastol, dehydroagastol, isoagastol were isolated [13, 14]. A new flavone glycoside was isolated from leaves of A. rugosa called acacetin 7-O-b-(600-(E)-crotonylglucopyranoside) [1]. However, agastinol and agastenol were reported to cause effect of protection against etoposide-induced apoptosis in leukemia cells U937 [14].

The main focus of *Agastache* species investigation is *A. rugosa*. Studies have showed that *A. rugosa* has many medicinal activities, such as antitumor, antiviral, antibacterial, antifungal properties, anti-inflammation, anti-atherogenic and anti-melanogenic activities [15-16]. Plant *in vitro* culture have been applied also mostly to *A. rugosa* for such purposes as micropropagation [7] and for studying regulation of the biosynthesis of specialized secondary metabolites [7, 17]. Efficient protocols for *in vitro* RA production have been developed for *A. rugosa* [18-19]. There is not enough information about other *Agastache* species in *in vitro* culture. Some reports were dedicated to *A. foeniculum* [20]. Plant cell cultures of other *Agastache* species could represent an available resource to obtain high-efficiency compounds it was already reported for *A. rugosa* and partly for *A. foeniculum* [7, 18-19]. Thus, the aim of this work was to develop efficient protocols for establishing callogenesis and cell suspension cultures for following phytochemical and pharmacological studies of three *Agastache* species – *A. foeniculum*, *A. scrophulariifolia*, *A. urticifolia*.

2 Materials and methods

2.1 Plant Material

Seeds were surface sterilized by immersion in 70% ethanol for 1 minute followed by 5% sodium hypochlorite solution for 15 minutes. Subsequently the seeds were rinsed three times with sterilized distilled water. The sterilized seeds were cultured on MS [25] medium without any growth regulators, containing 8 mg/L agar for solidification. A period of incubation of seeds amounted 60 days at 22 °C.

2.2 Callus Induction

For callus induction 0.5–1.0 cm segments of leaves and stems from 2 months old in vitro cultivated plants were used as explants. The explants were placed in Petri dishes on solid MS media (8 mg/L of agar supplement) supplemented with growth regulators. Cultures were maintained at 20 °C in the dark during eight weeks. For callus induction six variations of medium supplements were tested: 0.5 or 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2.4-

D) in combination with 0.1 mg/L kinetin (KIN); 1.0 or 2.0 mg/L 6-benzyladenine (BA) in combination with 0.1 mg/L α-naphthaleneacetic acid (NAA); 0.5 or 1.0 mg/L indole-3-acetic acid (IAA) in combination with 0.1 mg/L kinetin. Subcultures were executed at 28 days intervals.

2.3 Cell Suspension Culture

Friable calli after 6 months of culturing were used to obtain suspension cultures. To establish cell suspension culture 5 g of friable leaf- or stem-derived callus was carried to a 500 mL flask containing 50 mL of liquid MS or B5 media, supplemented with 1 mg/L 2.4-D, 0.1 mg/L KIN and 30 mg/L sucrose (pH 5.8). The suspension cultures were maintained at 22 °C on a shaker at 100 rpm in the dark. Cells were sub-cultured by transferring cell suspensions into fresh medium (1:2, v/v) with interval of 14 days. To retrieve data for the growth curves 14 days old inoculum cultures were used. Cell growth was defined at 3 days intervals during 1 month [26]. Also, growth of suspension cultures was monitored by cell counting using a hemocytometer [27].

For cells viability evaluation a counting method using the hemocytometer and Evans' blue was applied [27]. The percentage of unstained cells represents the percentage of viable cells in the suspension (% viable cells = viable cell number / total cell number). All results were received in triplicate.

2.4 Statistical Analysis

For callus induction, 20 explants were used for each treatment. Each experiment was performed in three independent replicates. Data were analyzed with ANOVA.

3 Results

Callus induction was achieved from explants within 6 weeks of inoculation.

A. foeniculum callus formation was observed on both types of explants for all types of nutrient medium. *A. foeniculum* stem explants showed the best result for all medium variations except the media with 0.1–0.2 mg/L BA (Table 1).

Table 1. Effects of explant types and combinations of growth regulators on *Agastache foeniculum* quality of calli and efficiency of callogenesis.

Supplements	Explant type	% of explants forming calli[1]	Callus quality[2]	Intensity of callus formation[3]
0.5 mg/L 2.4-D + 0.1 mg/L KIN	leaf	54.2 ± 2.9^{e}	Friable, pale green	++
	stem	100^{g}	Friable, pale green	+++
2 mg/L 2.4-D + 0.1 mg/L KIN	leaf	83.4 ± 9.7^{a}	Friable, pale brown	+++
	stem	83.4 ± 9.7^{a}	Friable, pale brown	+++
1.0 mg/L BA + 0.1 mg/L NAA	leaf	80.0 ± 2.7^{a}	Compact, brown	++
	stem	26.2 ± 1.8^{b}	Compact, green	+
2.0 mg/L BA + 0.1 mg/L NAA	leaf	36.1 ± 2.6^{d}	Compact, dark brown	+
	stem	0^{c}		-
0.5 mg/L IAA + 0.1 mg/L KIN	leaf	70.4 ± 3.8^{f}	Brown, heterogeneous	++
	stem	83.4 ± 7.8^{a}	Brown, heterogeneous	++
1.0 mg/L IAA + 0.1 mg/L KIN	leaf	27.0 ± 1.3^{b}	Brown, heterogeneous	+
	stem	86.3 ± 1.4^{a}	Dark brown, heterogeneous	++

[1] Values marking same letters indicate not significantly different according to Duncan test ($p < 0.05$); [2] Observation for primary callus subcultures; [3] + - low level; ++ - medium level; +++ - high level

Depending on the concentration and the combination of plant growth regulators the intensity of callus formation, its color and texture widely varied. The callus color and texture ranged from pale green to dark green, and from soft and friable to compact. At the concentrations 0.5 and 2.0 mg/L 2.4-D, both leaf and stem explants produced friable pale green or pale brown calli. On MS media supplemented with BA in concentration 1.0–2.0 mg/L in combination with 0.1 mg/L NAA, explants developed dark brown or green and compact calli. For media supplemented with 0.5 and 1.0 mg/L IAA in combination with 0.1 mg/L KIN heterogeneous dark brown calli were obtained. Maximum callus induction (100%) was achieved from stem explants for medium supplemented with 0.5 mg/L 2.4-D in combination with 0.1 mg/L KIN. The highest intensity of callus formation also was achieved for the media with 2.4-D. The callus from these media was selected for establishing cell suspension culture of *A. foeniculum* (Figure 1).

Fig. 1. *Agastache foeniculum* callus induction and establishing cell suspension culture. (a) intact plant; (b) seeds; (c) 60 days old *in vitro* plants; (d) 6 weeks old stem- and leaf-derived calli on MS medium supplemented with 0.5 mg/L 2,4-D and 0.1 mg/L KIN; (e) multiplication of friable stem-derived calli; (f) establishing cell suspension culture; (g) cell suspension aggregates (×10); (h) non-viable cell stained with Evan's blue (×10); (i) viable cells (×10).

For *A. scrophulariifolia* callus formation was observed on the media supplemented with 0.5–2 mg/L 2.4-D in combination with 0.1 mg/L KIN both from stem and leaf explants (table 2). Maximum callus induction (100%) was achieved from leaf explants (table 2). The explants formed compact white or pale green calli. For other variants of media callus formation was not observed.

Table 2. Effects of explant types and combinations of growth regulators on *Agastache scrophulariifolia* quality of calli and efficiency of callogenesis.

Supplements	Explant type	% of explants forming calli[1]	Callus quality[2]	Intensity of callus formation[3]
0.5 mg/L 2.4-D + 0.1 mg/L KIN	leaf	100.00[b]	Compact, pale green	+++
	stem	96.00 ± 1.48[d]	Compact, white	++
2 mg/L 2.4-D + 0.1 mg/L KIN	leaf	100.00[b]	Compact, white	+++
	stem	90.00 ± 2.13[c]	Compact, pale brown	++

[1] Values marking same letters indicate not significantly different according to Duncan test ($p < 0.05$); [2] Observation for primary callus subcultures; [3] + - low level; ++ - medium level; +++ - high level

For *A. urticifolia* callus formation was possible on the media supplemented with 0.5–2.0 mg/L 2,4-D in combination with 0.1 mg/L KIN, 1.0 mg/L BA in combination with 0.1 mg/L NAA for both explants' types, and 2.0 mg/L BA in combination with 0.1 mg/L NAA only for leaf explants (Table 3).

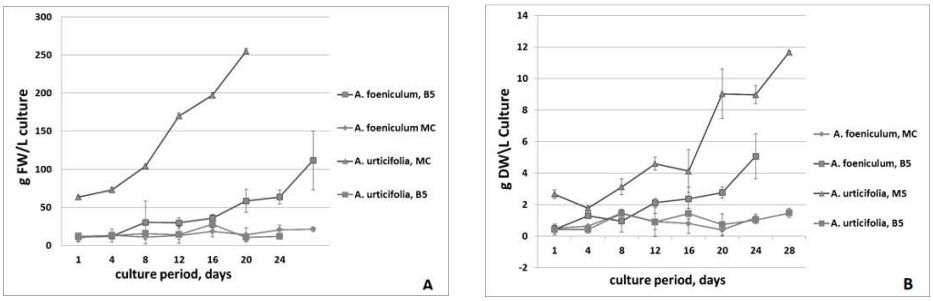
Table 3. Effects of explant types and combinations of growth regulators on *Agastache urticifolia* quality of calli and efficiency of callogenesis.

Supplements	Explant type	% of explants forming calli[1]	Callus quality[2]	Intensity of callus formation[3]
0.5 mg/L 2,4-D + 0,1 mg/L KIN	leaf	100.00[d]	Friable, pale green	+++
	stem	68.00 ± 11.43[b]	Compact, white	++
2 mg/L 2,4-D + 0,1 mg/L KIN	leaf	96.00 ± 1.65[cd]	Friable, pale green	++
	stem	84.00 ± 5.05[c]	Compact, white	++
1.0 mg/L BA + 0,1 mg/L NAA	leaf	64.00 ± 28.81[b]	Compact, green	+
	stem	34.00 ± 13.29[e]	Compact, green	+
2.0 mg/L BA + 0,1 mg/L NAA	leaf	60.00 ± 38.71[b]	Compact, green	+
	stem	0.00[a]		-

[1] Values marking same letters indicate not significantly different according to Duncan test ($p < 0.05$); [2] Observation for primary callus subcultures; [3] + - low level; ++ - medium level; +++ - high level.

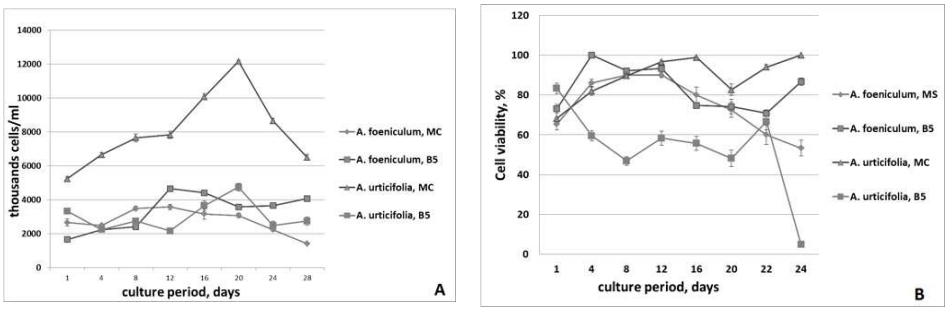
At concentrations 0.5–2 mg/L 2.4-D leaf explants produced pale green soft calli. At the same media stem explants established compact white calli. On the media supplemented with 1.0-2.0 mg/L BA in combination with 0.1 mg/L NAA both types of explants formed compact white or green calli. For media supplemented with 0.5–1.0 mg/L IAA in combination with 0.1 mg/L KIN callogenesis efficiency was zero.

For the establishing suspension cultures of all studied *Agastache* species soft friable calli generated on the medium supplemented with 0.5 mg/L 2.4-D in combination with 0.1 mg/L KIN were used. The calli were sub-cultured every 28 days onto a fresh medium, until obtaining homogenous callus lines after 6 months of culturing, which were used for the establishing of cell suspension cultures. The growth curves of *Agastache* cell suspensions based on fresh and dry weight quantification and cell amount in 1 mL suspension, are represented in Figure 2 and Figure 3 (a). Cell suspensions cultures of *A. foeniculum* entered the exponential phase during the 1st day of culture, until the 24th day with growth indexes of 1.8 (MS), 8.97 (B5) on a dry weight basis (growth index of the culture (final weight – initial weight)/initial weight). The percentages of cell viability varied between 89.87 and 30.26 for *A. foeniculum* suspension culture, 58.39 and 12.96 for *A. urticifolia* suspension culture (Figure 3 (b)) and 0 and 36.00 for *A. scrophulariifolia* suspension culture at the end of the 1st and 6th week, respectively. When the viability of cells remained about 50 %, it was considered that the suspension culture establishing was unsuccessful (Mathur & Shekhawat, 2013).



(a) (b)

Fig. 2. Growth curves of cell suspension cultures of *Agastache* species in liquid MS and B5 media supplemented with 1 mg/L 2,4-D and 0.1 mg/L KIN for 4 weeks of culturing: (a) FW – fresh weight; (b) DW – dry weight. The bars demonstrate the standard error (±SE) from three independent measurements.



(a) (b)

Fig. 3. (a) The cell number per mL suspension cultures of *Agastache* species cultured for 4 weeks; (b) the cell viability of *A. of Agastache* species suspension cultures. The bars demonstrate the standard error (±SE) from three independent measurements.

4 Discussion

The dependence of callus formation on explant type and plant growth regulators concentration was already reported for some *Lamiaceae* plants. For many *Lamiaceae* species calli were developed on the media supplemented with 2,4-D and BA mainly from leaf explants [21-22]. Optimal concentration of plant growth regulators may depend on many factors, such as a genotype of original plants, type of explants, its origin and age, mineral and composition of media [23].

In previous studies to the development of cultivation system to obtain RA and volatile components of essential oils in *A. rugosa* suspension culture, MS media containing 0.5–1.0 mg/L of 2,4-D were used [5,19]. We assume that the MS medium supplemented with 1 mg/L 2.4-D and 0.1 mg/L KIN is not suitable for establishing suspension cultures of *A. urticifolia* and *A. scrophulariifolia*. We plan to use other variation of nutrient media for these aims, for example B5 medium. In cell suspension culture of *A. rugosa* for RA production the maximum growth (7.7 g/L) was achieved in the liquid B5 medium supplemented with 2 mg/L 2,4-D and 0.1 mg/L BA [18]. For some *Lamiaceae* species, such as *Satureja hortensis* L. [24], *Satureja khuzistanica* [22] B5 medium also was preferable for

obtaining cell suspension cultures. Consequently, we suppose that the use of B5 medium could be effective for establishing cell suspension cultures of other *Agastache* species. Mineral compound of media, particularly NH_4^+ and NO_3^- concentrations and source influence phenolic secondary metabolites production of *Lamiaceae*, especially RA, and biomass accumulation, which was confirmed in some studies [22].

Carbon source and concentration are important factors of cell suspension culture cultivation. Growth of suspension culture of *A. rugosa* was improved, when it was cultivated in MS medium supplemented with 50 mg/L sucrose [5]. Thus, an increase in the sucrose content in the medium can be used to improve the cell growth of *Agastache* suspension cultures.

5 Conclusion

In result, for the first time a suitable cell suspension culture was established with soft stem-derived callus of *A. foeniculum* as a background for further studies on the production of secondary metabolites *in vitro*. Obtaining a stable suspension culture is the first step to develop a system for valuable secondary metabolites production *in vitro*. The resulting culture showed sustainable and stable growth throughout the entire cultivation period and high viability with a maximum of 89.87% on the 8-12 days of cultivation. This study shows prospects of using not only *A. rugosa*, but also *A. foeniculum* for the development of biotechnological systems to synthesize pharmaceutically active components using plant cells and tissue culture. For *A. urticifolia* and *A. scrophularifolia* efficient cell suspensions were not established. The cell viability of these two species in suspension culture was low during all period of cultivation did not exceed 36 % for *A. scrophulariifolia* and 58,39 % for *A. urticifolia*. When cell viability remained around 50 %, it was considered that the suspension culture establishing has failed. For *A. scrophulariifolia* too hard and compact calli were developed. This type of callus is not suitable for establishing suspension culture. To obtain stable suspension cultures of these species, it is necessary to optimize protocols for callus induction of and to take into account factors of cultivation, such as composition of media.

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