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MICROPROPAGATION OF *AGASTACHE ANISATA* USING NODAL SEGMENTS AS EXPLANTS AND CYTOTOXIC ACTIVITY OF ITS METHANOLIC EXTRACTS

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Abstract

Agastache anisata (anise hyssop) is an herb grown for ornamental and culinary purpose. The present study focuses on the direct regeneration efficiency of *Agastache anisata* from nodal segments and subsequent acclimatization of the regenerated plants. Plant shoot organogenesis and multiplication was found at its maximum on Murashige and Skoog medium supplemented with 0.1 mg/L of 6-Benzyl amino purine (BAP) and 0.05 mg/L of Indole-3-acetic acid (IAA). Furthermore, same medium was found effective for the induction of roots, in the *In vitro* grown plantlets. Among the various pot mixes used for the acclimatization of rooted plantlets, equal proportion of sand and farmyard manure was found to be the best supporting material for acclimatization. The methanolic extract of *Agastache anisata* was evaluated and showed mild cytotoxic activity. This is the first report on *in-vitro* multiplication of *A. anisata* through plant tissue culture.

Introduction

Horticultural research on medicinal plants has focused on developing the capacity for optimal growth in cultivation. This has been especially pertinent as many medicinal plants are still harvested in the wild (Briskin, 2000). Bringing herbs into cultivation, traditional and biotechnological plant-breeding techniques can be applied at the genetic level to improve yield and uniformity, and to modify potency or toxicity (Canter *et al*., 2005). Plant tissue culture is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Khan & Bibi, 2012; Rout *et al*., 2000).

Agastache anisata is a hardy perennial herb native to the United States belongs to genus *Agastache*. The genus Agastache (Lamiaceae) includes 22 species of aromatic herbaceous perennials (Fuentes-Granados *et al*., 1998) spread over most of the United States and extends to Central Mexico and Canada. One species also occurs in Japan, Manchuria and Eastern China (Lint & Epling, 1945). The leaves and blooms of *A. anisata* are highly fragrant and smell like licorice candy. It is winter hardy; being one of the hardiest agastache plants, these lavender blooms to any garden setting for a cool splash of color (Anonymous, 2011). Estragol is the main constituent of this plant (Burzo *et al*., 2006), which has limited antibacterial activity (Bagamboula *et al*., 2004). This species has not been studied extensively, which makes it even more appealing for its *in-vitro* exploitation, as other species of Agastache have exhibited good medicinal and culinary properties (Ayers & Widrlechner, 1994; Charles *et al*., 1991; Fuentes-Granados *et al*., 1998; Shin & Kang, 2003).

Since this species have not been studied extensively, thus bioassay studies were conducted. The brine shrimp lethality test (BST), developed by Meyer *et al*., (1982) is being used as a simple tool to guide screening and fractionation of physiologically active plant extracts (Zaidi & Crow Jr., 2012). This general bioassay detects a broad range of biological activities and a diversity of chemical structures. However, it has been demonstrated that BST correlates reasonably well with cytotoxic and other biological properties (McLaughlin, 1991). Brine shrimp have been previously utilized in various bioassay systems (Hameed *et al*., 2013).

To date, this is the first report on *in-vitro* multiplication of *A. anisata* through direct plant regeneration technique, which offers an effective alternative method for propagation of this plant. The presented protocol offers adequate understanding regarding the micropropagation of *A. anisata* and also aid in studying various aspects of this prospective medicinal plants' secondary metabolites production and biotransformation studies.

Material and Methods

Explant: Seeds of *A. anisata* (Code 7785, Thomson & Morgan, USA) were surface sterilized using a method by Khan *et al*., 2008. These surface sterilized seeds were planted onto Murashige & Skoog (1962) culture medium (MS) supplemented with 3% sucrose, 100 mg/L myo-inisitol, and 6 gm/L agar (pH 5.75) for germination.

Shoot induction and multiplication: Under aseptic conditions, nodal segments (1-2 cm) were excised from previously grown *A. anisata* seedlings (21 days old) and cultured on MS medium (Murashige & Skoog, 1962) used for shoot induction and multiplication, containing 3% (w/v) sucrose, supplemented with different concentrations and combinations of BAP and kinetin (KN) (0.01, 0.05, 0.5 and 1.0 mg/L) [Table 1]. The pH was adjusted to 5.75 prior to the addition of 0.6% agar and autoclaved at 121°C, 15 p.s.i., for 15 minutes. Fifteen replicates of each shooting media formulation were inoculated. Cultures were then incubated at 25±2°C. Data were recorded weekly for Shoot regeneration, number of shoots, and shoot length up to five weeks and values are the means ± standard deviation.

Root induction: *In vitro* rooting in *A. anisata* was studied using MS media with different types of auxins like IAA and IBA (Indole-3-butyric acid), at the concentrations of 0.01, 0.05, 0.5 and 1.0 mg/l. A simple MS medium without any growth regulators was used as a control. In order to check synergistic effect, IAA and IBA were also applied in combination with BAP 0.1 mg/L (Table 1). All the media formulations for root induction had 2.5% sugar, and 6 gm/L of agar as a solidifying agent. The media were sterilized in an autoclave, with a pH 5.75 prior to autoclaving. Fifteen explants (obtained from the shoot induction and multiplication experiment) were cultured onto each formulation. Data were recorded weekly for five weeks, and values presented as means ± standard error.

Table 1. Effect of different concentrations plant growth regulators in MS medium for direct regeneration of *Agastache anisata*.

Code	Growth regulators				Shoot regeneration% per explant	No. of shoots	Average shoot length	% Root formation	No. of roots	Average root length
	BAP	Kn	IAA	IBA						
M0					54	4.20±0.8	2.06±0.34	67	4.46±0.72	2.93±0.47
M1	0.05				87	5.26±0.55	2.87±0.21			
M2	0.1				100	7.66±0.12	4.06±0.07			
M3	0.5				100	7.67±0.18	3.90±0.05			
M4	1				100	7.80±0.20	3.37±0.10			
M5		0.05			67	2.46±0.36	1.12±0.20			
M6		0.1			74	3.06±0.41	1.36±0.19			
M7		0.5			80	4.06±0.44	1.50±0.15			
M8		1			87	4.93±0.37	1.28±0.07			
M9			0.05		100	7.66±0.15	1.51±0.04	94	4.94±0.17	3.89±0.20
M10			0.1		100	7.63±0.12	1.46±0.06	100	5.48±0.15	4.60±0.16
M11			0.5		94	7.60±0.13	1.44±0.07	100	5.50±0.14	4.56±0.11
M12			1		94	7.53±0.16	1.42±0.08	100	6.06±0.11	4.50±0.14
M13				0.05	87	-	-	87	4.46±0.16	2.60±0.13
M14				0.1	74	-	-	87	4.33±0.18	2.56±0.18
M15				0.5	54	-	-	94	4.30±0.23	2.33±0.11
M16				1	54	-	-	100	4.20±0.22	2.00±0.21
M17	**0.1**		**0.05**		**100**	**7.93±0.06**	**5.97±0.18**	**100**	**5.96±0.04**	**4.01±0.01**
M18	0.1		0.1		100	7.80±0.17	5.91±0.07	100	5.94±0.10	3.98±0.09
M19	0.1		0.5		100	7.78±0.25	5.55±0.13	100	5.86±0.07	4.01±0.21
M20	0.1		1		94	7.69±0.11	5.40±0.13	100	5.79±0.11	4.04±0.38
M21	0.1			0.05	94	4.63±0.36	1.90±0.07	87	3.99±0.13	2.16±0.16
M22	0.1			0.1	87	3.80±0.41	1.78±0.09	94	4.66±0.21	2.93±0.06
M23	0.1			0.5	74	3.13±0.35	2.88±0.09	100	4.60±0.21	2.10±0.13
M24	0.1			1	67	2.26±0.31	2.73±0.15	100	4.86±0.16	2.73±0.15

15 replicates were maintained in each treatment and data were recorded up to four weeks of culture. Values are means ± Standard Error (SE)

Acclimatization: Different pot mixes were tried to select appropriate planting media for the *ex vitro* growth of plantlets. An equal number of plantlets were transferred to each pot containing various mixes, and their survival rate was calculated by collecting data after three months (Table 2). Combinations exhibiting survival rate of 60% or above are mentioned only.

Brine shrimp lethality assay: Leaf sample (5 gm) was cleaned, dried in an oven at 60°C, powdered and extracted using 80% methanol (20% water). The extracts were filtered using Whatman No. 1 filter paper. A rotary evaporator was used for removal of the solvent. Residues obtained were dried at room temperature prior to assay.

For bioassay, half filled hatching tray (a rectangular dish (22 x 32 cm) with brine solution (sea salt 38 g/L of d/w) containing 500 mg eggs of brine shrimp (*Artemia salina*) were sprinkled and covered with a lid, followed by incubation at 27°C for 2 days for hatching. The brine shrimp larvae were collected through a light source and Pasteur pipette. The sample (20 mg) was dissolved in 2 mL of solvent (DMSO) and then 500 µL, 50 µL and 5 µL of this solution was transferred to vials corresponding to 1000, 100 and 10 µg/mL respectively and was evaporated overnight. After two days of hatching, larvae were placed in each vial using a Pasteur pipette. The volume was raised to 5 mL with syringe, by adding seawater and incubated at 27°C for 24 h under illumination. Etoposide was used as a standard reference cytotoxic drug. After 24 hr, the number of survivors were counted and recorded. The data were analyzed with Finney computer program to determine LD50 values at 95% confidence intervals.

Results and Discussion

A highly efficient protocol for the direct regeneration and acclimatization of *Agastache anisata* was established. The protocol is cost effective as rooting was performed on the same medium that was used for shoot multiplication.

The cultured nodal segments showed sprouting within four days (Fig. 1a). MS medium individually supplemented with both BAP and Kinetin (KN) showed notable response. Among cytokinins, BAP responded well as compared to KN for shoot proliferation (Table 1). The maximum induction of multiple shoots (7.66±0.12 cm) was achieved on medium supplemented with 0.1 mg/L BAP (M2), four weeks after incubation, with an average shoot length of 4.06 ± 0.07cm (Fig. 1b,c). Although higher concentrations (M4) did produce more shoots, but the corresponding shoot lengths were on gradual decrement (3.37 ± 0.10 cm). The effect of kinetin was also much less as compared to BAP in shoot elongation and the plantlets did not attain full length as well (Table 1).

Table 2. Effect of different potting mixes on acclimatization of *Agastache anisata.*

Potting mix	Code	Survival rate* (%)	Leaf color/Morphology
100 % Sand	Ac1	90	Light green/Normal
75 % Sand + 25 % FYM	Ac2	90	Green/Normal
50 % Sand + 50 % FYM	**Ac3**	**95**	**Green/Normal**
25 % Sand + 75 % FYM	Ac4	85	Green/Normal
50 % Sand + 50 % Coconut Husk	Ac5	70	Light green/Stunted
50 % Sand + 50 % Grinded Charcoal	Ac6	60	Light green/Stunted

* Values are rounded off

Fig. 1. Different stages of Micropropagation of *Agastache anisata*. (a) Nodal segments show sprouting on 4th day. (b) Shoot multiplication (c) Root induction. (d) Acclimatized plant.

The regenerated shoots reached a length of more than 3-4 cm after four weeks. They were separated and planted on MS basal medium with IAA (M9-12) and IBA (M13-M16), alone and also in combination with BAP 0.1 mg/L i.e., optimized cytokinin (M17-M24) in order to evaluate the synergistic effect of cytokinin:auxin for direct organogenesis. IAA combinations responded well as compared to IBA, though NAA (1-Naphthalene acetic acid) was also tested, but the results were insignificant and mostly induced callus (data not shown). In cultures, where the shoots were inoculated on auxin-supplemented medium only (M9-M16), root primordia emerged from the shoot base on first week of culture. Each combination induced roots in the plantlets but the plant growth became undersized, albeit M12 produced more roots (6.06±0.11) than any other medium. On the other hand, in M17 medium (IAA 0.05 mg/L and BAP 0.1 mg/l), number of roots induced were 5.96±0.04, the medium showed best response for shoot multiplication (7.93±0.06) and shoot length (5.97±0.18) of the plant (Fig. 1b,c), far better than any other media formulation. Since, it is a known fact that IAA is an auxin that got oxidized and metabolized rapidly, aiding in shoot and embryo formation, low concentration of IAA promotes root growth and maintains the apical dominance (George, 1993). Also, earlier reports suggest that related familial species like *Ocimum* has shown a good regeneration capacity in MS medium in the presence of BAP combined with auxins (Dode *et al*., 2003; Phippen and Simon, 2000). The results are illustrated graphically (Fig. 2) where it is comparably evident that as opposed to others, single culture medium (M17) is better when four growth parameters altogether are taken into account. The other best medium was M18, which showed results similar to M17, but the later was preferred due to low amount of PGRs. The axillary buds on further multiplication survived well and developed as individual plants.

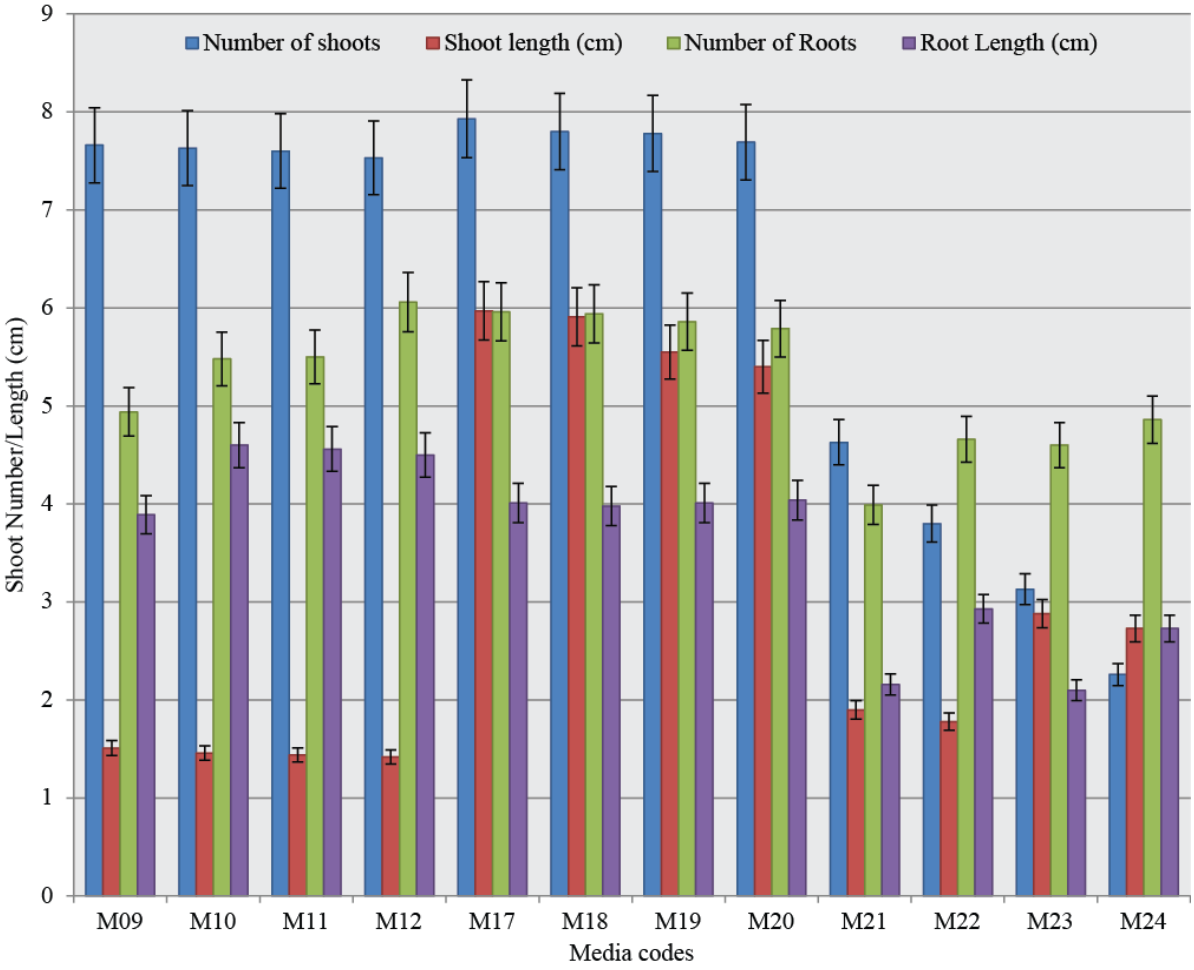

Fig. 2. Comparison of different media formulations with optimized culture medium (M17).

For acclimatization, rooted plantlets were removed from the rooting medium after four weeks of culture, and transferred onto plastic pots containing four different combinations of potting mixes (Table 2) in a green house. The viability of the plantlets was observed weekly, and the survival rates of the *In vitro* grown plantlets were calculated after three months (combinations that have ≥ 60% survival are mentioned). A high survival rate (95%) was observed in 50:50% of sand and FYM (Table 2). Only Ac3 combination matched the normal growth pattern of *A. anisata* in terms of length and leaf color (Fig. 1d). The respective survival rates of the plantlets in different potting mixes are presented in Table 2.

It was concluded that although the plantlets were sufficiently rooted, it is crucial for the plantlets that they should be kept in the tunnel covered with the plastic sheet, prior to the transfer of the plantlets under green house conditions. However, as the cuticle layer of plant developed gradually, the plants become more resistant to environmental stresses (Khan *et al.*, 2007).

In brine shrimp lethality test, we test the toxicity of *A. anisata* methanolic extract. Although the toxicity of etoposide is higher than the *A. anisata* extract, but if 100 µg/mL is regarded as an approximate border line for toxicity, the extract exhibit low cytotoxic activity on brine shrimp larva (Table 3). In this study, only the best result is being considered, excluding other tissue sources and organic solvents yielding non-significant result. Considering that a challenge today is the discovery of plants with promising activities, we have applied in this work the brine shrimp test (BST) for general activity screening of plant extract.

Table 3. Cytotoxic activity of *Agastache anisata*.

Dose (µg/mL)	Number of shrimps	Number of survivors	LD_{50} (µg/mL)	Standard Drug	LD_{50} (µg/mL)
1000	30	11			
100	30	20	385.02	Etoposide	7.4625
10	30	24			

Conclusion

Direct shoot multiplication is preferred for generating true-to-type plants than callus regeneration. This study supports the rapid multiplication of this useful medicinal plant by *in-vitro* conditions. This report provides a simple protocol for the micropropagation of *Agastache*. Shoots can be easily derived from node cultures, and subsequently to rooting on BAP supplemented with IAA containing medium. This study offers an approach for producing identical plantlets from nodal explant of *Agastache* and its mild activity encourage working on the isolation of compounds from this plant.

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