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In Vitro Propagation of *Eryngium Foetidum* L. Via Callus Induction And Multiple Shoot Formation

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Abstract
A reliable and straightforward procedure for in vitro propagation of *Eryngium foetidum* was established using nodal and leaf explants. This study was conducted to evaluate the consequences of various growth regulators on callus induction and multiple shoot development. Callus induction was achieved at a rate of 100% when the medium was supplemented with IAA (5.7 µM). Meanwhile, multiple shoots were produced at an average of 6 ± 0.31 per plantlet when the medium was supplemented with a combination of BA (1.11µM) and Kinetin (1.16 µM). Following this, the plantlets were successfully hardened and acclimatized in the field with 70% survival rate.

Keywords: in vitro propagation, callus induction, nodal segments, *E. foetidum*, multiple shoots, Growth regulators.

Introduction

Eryngium foetidum is a perennial herb, belongs to the family Umbelliferae. It is used in folklore medicine and also as a substitute for coriander due to its coriander-like scent (Yasseen 2002). It is aromatic, and contains 0.1 – 0.9% of essential oils. The essential oils obtained from this plant species have high commercial value in international trade for their application in the perfumery and pharmaceutical industries (Ignacimuthu et al., 1999). The leaves of E. foetidum have been reported to possess anti-inflammatory and analgesic properties (Saenz et al., 1997). The root of the herb is sticky, nervine tonics, aphrodisiac, expectorant diuretic, diaphoretic, and stomachic. The leaves and roots of the plant are used in the treatment of gastrointestinal problems, and the leaf paste is used as an antiseptic agent by the ethnic communities residing in the forests of Karnataka state, India (Lingaraju et al., 2016).
The leaves of *E. foetidum* are commonly used as a condiment in North-Eastern India (Sankat and Maharaj 1996) and are also pickled in Sikkim. The plant has been employed as cattle fodder in Java (Burkill 1995). It is one of the plant species utilized in a Japanese patent for developing a skin-whitening agent (Paula et al., 2011). *E. foetidum* is used as a substitute for *Coriandrum sativum* as a food flavouring agent (Martin 2003). The herbs of *E. foetidum* have been widely utilized in various traditional systems for their health benefits. Based on traditional knowledge, several biologically active components have been isolated and identified (Kaushal and Yadav 2014).
E. foetidum can be propagated in vitro through micropropagation, which is a valuable technique for whole plant regeneration from explants. Although there have been few reports on in vitro regeneration of *E. foetidum* (Ignacimuthu et al., 1999, Arockiasamy et al., 2002), the present study aimed to standardize a simple protocol for in vitro propagation of *E. foetidum* from nodal and leaf explants.

Materials and Methods

Actively grown young leaves and nodal segments of *E. foetidum* were collected from Narikkuni, Calicut, Kerala were used in this study. The surface sterilization of explants was standardized using 70 % ethanol for 10 seconds and with NaOCl (0.6 % w/v) for 2 minutes followed by rinse with sterile distilled water. After sterilization, the explants were trimmed to about 1 cm (for nodal explant) by cutting its upper and lower end to remove the dead cells which were in direct contact with the sterilization agents. For leaf explants, the dead cells were trimmed and inoculated on the sterile culture medium.

Murashige and Skoog (1962) basal medium with Growth regulators such IAA (1.45, 2.9, 5.7 and 11.4 µM), 2, 4-D (1.13, 2.26, 4.52 and 9.03 µM), BAP (1.11, 2.22, 4.44 and 8.88 µM) and Kinetin (1.16, 2.32, 4.64 and 9.29 µM) both individually and in combination were used. For the preparation of media only analytical reagents of 'Hi Media' grade chemical and Borosil glassware were used. De-ionized water was used for preparing the media. The nutrient media basically consists of inorganic nutrients, carbon sources, and vitamins. Stock solutions were prepared separately for macronutrients, micronutrients; iron EDTA, potassium iodide and, vitamins. All the chemicals were weighed accurately in an electronic weighing balance. All stock solutions were preserved in a refrigerator at 4°C. The final volume was made up with distilled water and the pH of the medium was adjusted to 5.6 with either 1N NaOH or 1N HCl. To the

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above-said media, 0.8 to 0.9% agar (extra pure gelling point 32-35°C Hi-Media) was added and melted in a water bath. Around 10-15 ml of the medium was dispensed into 250 mm × 150 mm culture tubes.

The mouth of the tubes was covered with aluminium foil and was autoclaved at 1.06 kg pressure for about 20 minutes at 121°C. The autoclaved medium in the culture tubes was cooled and allowed to solidify as slants. The inoculations were done after four days to ensure that the media were free from contamination. Five replicates were used for each treatment maintained at 25±2°C in the culture room under a 12 h photoperiod of 50 µmol m^{-2} s^{-1} irradiance provided by white fluorescent tubes and with a relative humidity of 70%. For acclimatization, the plantlets with well-developed roots (3 to 5 cm) were removed from culture tubes, washed with sterile distilled water to remove the remnants of agar and plantlets were planted separately one to 10 cm diameter poly cup filled with potting mixture river sand, and garden soil (1:2).

All experiments were found out during a completely randomized design. Data presented in the table are treatments of 10 replicates (culture tubes) and all experiments were repeated thrice. Data were analysed and expressed in terms of Mean values ±SE and recorded.

Results and Discussion

Leaf segments of *E. foetidum* were cultured on MS media supplemented with different concentrations of 2,4-D and IAA for callus induction. The basal medium without plant growth hormones was used as a control. Compact calli were formed from leaf within 40 days (Figure 1 B&C). While there was no response in the control, calli formed from the leaf segment shows different frequencies (Table 1). Among tested treatments, IAA shows the callus induction in *E. foetidum* leaf explants.

The maximum frequency (100%) of callus formation was observed on the medium containing IAA (5.7 µM) whereas the medium supplemented with 2,4-D doesn't support the callus formation. IAA proved to be more effective than 2,4-D in callus induction from leaf explants of *E. foetidum*. The results indicate the important role of exogenous plant growth regulators on callus formation in *E. foetidum*.

Previous literature also reported that the organogenesis on *E. foetidum* was achieved through induction of shoots from ex vitro leaf-derived callus and root explants on a sucrose-supplemented medium fortified with IAA and BA. Therefore, IAA was generally more effective than Kinetin and IAA combination in inducing shoots from the callus, as has been shown in several medicinal plant species, including *Lippia alba* (Gupta et al., 2007), *Hemidesmus indicus* (Sreekumar et al., 2000), and Holostemma ada-kodien (Martin 2002).

The efficacy of exogenous IAA found in this experiment was also been reported with other medicinal plants by various authors. However, the results of this study are in agreement with the use of synthetic plant growth regulator in the medium for callus induction of *Withania somnifera* (Rani et al., 2003), *Cardiospermum halicacabum* Linn. (Thomas and Maseena, 2006) and *Vitex trifolia* (Sanghamitra et al., 2013). In *V. trifolia*, calli were initiated from stem, petioles, and leaves within 18-20 days of culture on MS basal medium supplemented with different concentrations of hormones.

Table 1. Effect of plant growth regulators on callus induction of *E. foetidum*

Hormone (µM)		Number of days taken for response	Frequency of callus induction (%)
2,4,D	IAA		
2.26	-	-	-
4.52	-	-	-
9.03	-	-	-
	2.9	40	20
	5.7	**40**	**100**
	11.4	40	-

Only the significant treatments are computed here
Data represents the mean of ten replicates

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**Figure 1: Callus induction in *E. foetidum*.
A: Leaf inoculation. B&C: callus formation.**

The multiple shoot formation using nodal segments has proved to be an efficient method for mass multiplication of plants. In the present study, two experimental setups were performed to find out the best concentration and combination of growth regulators. The first experiment is to culture the nodal segments of *E. foetidum* inoculated on MS medium supplemented with different concentration of BA, IAA, and Kinetin (Table 2), alone and Whereas in the second experiment, a combination of different growth regulators (Table 3) with the best concentration in experiment 1.
MS medium containing both the cytokinin and auxin alone supported the growth of multiple shoots. The highest frequencies (100 %) of multiple shoots were found in MS medium supplemented with BA alone (1.11 µM) after 18 days. It induces multiple shoots at an average maximum of 3.3 ± 0.13 shoots with an average height of 1.4 ± 0.06 cm and also has 2.4 ± 0.18 average numbers of roots with 1.06 ± 0.06 cm root length. When the MS medium supplemented with the combination of cytokinin and auxin also promoted maximum multiple shoot and roots. The maximum frequencies of 80 % of multiple shoots were found in the medium supplemented with both BA (1.11 µM) and kinetin (1.16 µM). It shows a 6 ± 0.31average number of the shoot with 1.2 ± 0.37 cm plantlet height and also has 4 ± 0.44 average numbers of roots with 0.5 ± 0.14 cm root length (Figure 2C-E). The importance of BA for regeneration of different plants for example *Ocimum* spp. has been emphasized by Dode et al. (2003), Nirmal and Sehgal (1999) and David and Arockiasamy (2008), Kiran et al. (2004) showed that MS medium supplemented with 1 mg/L BAP was effective for shoot multiplication in the nodal segment of *Mentha piperita* L. In our study also the concentration of BA supported the good multiple shoot induction instead of another cytokinin, Kinetin. Well, rooted plantlets were separated and hardened with a 60 % survival rate (Figure 2F).

Table 2: Effect of hormones alone on multiple shoot induction in *E. foetidum*

Hormone (µM)			Number of days taken for multiple shoot induction	Frequency of multiple shoot induction (%)	Average number of shoots (±SE)[a]	Average shoot height (cm) (±SE)[a]	Average number of roots (±SE)[a]	Average root length (cm) (±SE)[a]
BA	Kinetin	IAA						
1.11			**18**	**100**	**3.3 ± 0.13**	**1.4 ± 0.06**	**2.4 ± 0.18**	**1.06 ± 0.06**
2.22			18	60	1.4 ± 0.57	1 ± 0.41	1 ± 0.44	0.5 ± 0.2
4.44			18	60	1.6 ± 0.65	0.6 ± 0.25	1.25 ± 0.51	0.5 ± 0.2
8.88			18	60	1.4 ± 0.58	1 ± 0.47	1 ± 0.45	0.6 ± 0.25
	1.16		45	60	3 ± 0.76	0.5 ± 0.21	0.7 ± 0.43	0.6 ± 0.25
	2.32		18	40	2.6 ± 0.74	0.75 ± 0.45	8.5 ± 0.07	1.25 ± 0.76
	4.64		18	20	0.8 ± 0.48	0.6 ± 0.4	1 ± 0.63	0.4 ± 0.4
	9.29		18	40	1 ± 0.61	0.65 ± 0.39	6 ± 0.07	1.5 ± 0.67
		1.45	18	40	1.6 ± 0.74	0.55 ± 0.33	1.4 ± 0.85	0.4 ± 0.24
		2.9	18	20	1 ± 0.63	0.5 ± 0.31	1 ± 0.61	0.5 ± 0.3
		5.7	18	-	-	-	-	-
		11.4	18	-	-	-	-	-

Only the significant treatments are computed here

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Data represents the mean of ten replicates
[a] ± SE: Standard error

Table 3: Effect on combination of growth regulators on multiple shoot induction in *E. foetidum*

Hormone (µM)			Number of days taken for multiple shoot induction	Frequency of multiple shoot induction (%)	Average number of shoots (±SE)[a]	Average shoot height (cm) (±SE)[a]	Average number of roots (±SE)[a]	Average root length (cm) (±SE)[a]
BA	Kinetin	IAA						
1.11	**1.16**		**21**	**80**	**6 ± 0.31**	**1.2 ± 0.37**	**4 ± 0.44**	**0.5 ± 0.14**
1.11	2.32		21	40	3.2 ± 0.86	1 ± 0.61	3 ± 0.75	0.7 ± 0.45
1.11	4.64		21	20	2.1 ± 0.86	0.8 ± 0.48	1 ± 0.61	0.2 ± 0.12
1.11	9.29		21	60	3.6 ± 0.50	1 ± 0.44	2 ± 0.83	0.5 ± 0.20
1.11		1.45	21	40	3 ± 0.79	0.65 ± 0.39	2 ± 0.83	0.4 ± 0.24
1.11		2.9	21	20	1 ± 0.63	0.5 ± 0.31	2 ± 0.82	0.5 ± 0.31
1.11		5.7	21	20	2 ± 0.88	1 ± 0.63	2 ± 0.83	0.5 ± 0.3
1.11		11.4	21	20	1 ± 0.63	0.6 ± 0.36	2 ± 0.85	0.5 ± 0.31

Only the significant treatments are computed here
Data represents the mean of ten replicates
[a] ± SE: Standard error

**Figure 2: Multiple shoot induction in *E. foetidum*.
A: Nodal explant. B: Shoot initiation.
C-E: Multiple shoot formation. F: Acclimatized plantlet**

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