Indian Journal of Plant Sciences ISSN: 2319–3824(Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jps.htm
2015 Vol.4 (2) April -June, pp.52-58/Chandrika et al.
Research Article

REDUCTION OF VITRIFICATION IN *IN VITRO* SHOOT CULTURES OF *ERYNGIUM FOETIDUM* L. - A POTENTIAL AROMATIC AND MEDICINAL HERB

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ABSTRACT
A protocol is detailed to reduce the vitrified structures in *in vitro* shoot cultures of Eryngium foetidum L., a potential aromatic and medicinal herb. Multiple shoots were produced on Murashige and Skoog medium supplemented with various concentrations of NAA, BAP and kinetin using nodal and scape explants. The vitrified structures initiated during 8-12th days of culturing in the medium. They increased rapidly in number and transformed into various configurations after 14-15 days. A decrease in the concentration of specific kinetins coupled with the addition of adjuvants like coconut milk, phloroglucinol and casein hydrolysate decreased the incidence of vitrification. The vitrified structures also reduced with increase in the concentration of the gelling agent (agar agar) and controlling the relative humidity. The healthy shoots when transferred to soil showed upto 95% survivability. The histological studies clearly indicated the differences in the anatomical structures of vitrified and normal shoots.

Keywords: *Eryngium Foetidum L., Vitrification, Coconut Water, Phloroglucinol, Casein Hydrolysate*

INTRODUCTION
Eryngium foetidum L.(Culantro), belonging to the genus *Eryngium* in the family Apiaceae, is a potential aromatic and medicinal herb, popular as a condiment among the ethnic populations of the Caribbean, Amazon, IndoChina and South east Asia (Adams, 1956; Ignacimuthu,1991). The plant has originated in South America and Amazon where wide range of therapeutic properties like treating cold, cough, seizures, stomach ailments, diabetes, hypertension, malaria and elevated cholesterol levels (Rajith *et al.*, 2010; Paul *et al.*, 2011). The extract of the plant rich in 2-E-Dodecenal has been patented for its antihelminthic property (Forbes *et al.*, 2009). The plant has also been known for its analgesic, anti-inflammatory and anticlastogenic properties (Saenz *et al.*, 1997; Mekhora *et al.*, 2012; Promkum *et al.*, 2012). The essential oil distilled from the leaves of the plant is a complex mixture of about 40 compounds and is indispensable in perfumery industry (Wong *et al.*, 1994; Pino *et al.*, 1997).
Of late there has been a `great demand for the herb owing to its strong coriander-like aroma which suits the preparation of a number of dishes in continental cuisine (Worz *et al.*, 2010). A wider awareness about its aromatic and medicinal value has made this herb very attractive at the International markets (Ignacimuthu *et al.*, 1991). However its wide adaptability as a commercial crop in cultivation is hindered because of a low germination rate which is erratic and allochronic followed by a slow growth phase (Chandrika R *et al.*, 2013). The other constraint being a lack of adequate quantity of seeds required for its large scale agronomy (Mozumder SN *et al.*, 2013). The natural populations of the plants which are rare and endemic hence face a huge threat due to increasing requirement of the herb for various purposes.
Vitrification (Hyperhydricity) of shoots under *in vitro* culture conditions is a common phenomenon that directly affects the net yield of multiple shoots. It is a complex disorder arising as a result of numerous culture conditions and affects most of the *in vitro* micropropagation procedures (Kevers *et al.*, 1984; Leshem *et al.*, 1988). The present study deals with the various approaches involved in modifying the ingredients of the culture medium so as to reduce the occurrence of *in vitro* vitrification and obtain maximum healthy shoots of *E. foetidum* under optimum culture conditions. Therefore an efficient *in vitro* conservation strategy (*ex situ* method) has been adapted and established as an essential part of our

research work. This communication is also the first report about the incidence and remedial measures for *in vitro* vitrification of *E. foetidum* cultures.

MATERIALS AND METHODS

Explant preparation: The scape and nodal explants about 12-14mm in length were excised from a 12-15 d old inflorescence and used for clonal propagation. These explants were thoroughly cleaned under a running tap for 15 min followed by a soak in Tween -20 detergent for 5 min. They were then pretreated with 0.1% streptomycin sulphate and 0.2% Bavistin for 15 min. Surface sterilization of the explants was carried out in 70% alcohol and 0.1% mercuric chloride for 5-8 min and finally rinsed with sterile distilled water for 5-6 times prior to culturing.

Media and culture conditions: The Murashige and Skoog medium (Murashige and Skoog, 1962) was prepared and supplemented with 6-benzyl amino purine (BAP) and Kinetin in various concentrations ($0.5 mgl^{-1} – 2.0 mgl^{-1}$). The media was fortified with coconut water (10%), Phloroglucinol (2-10 mgl^{-1}) and casein hydrolysate (5-15 mgl^{-1}) as a remedial measure to eliminate vitrifications in the culture. The P^H of the medium was adjusted to 5.8 before adding the gelling agent agar at a concentration of 8-12 g l^{-1}. Sterilization of the media was carried out at 1.06 kg cm^{-2} pressure for 20 min.

The explants were cultured onto the above MS medium containing various plant growth regulators and adjuvants. The culture tubes (53mmx125mm; 2mm thick) and the culture bottles (25mmx200mm) used for culturing were closed with polypropylene autoclavable screw capped lids to maintain the proper relative humidity. All cultures were incubated at $23±2^0$C with 16/8 h photoperiod at a photosynthetic photon flux of 25µmol $m^{-2}s^{-1}$.

Transfer to field conditions: After 4 weeks in culture, the healthy, rooted shoots were transferred to small pots containing sand and soil (1:1) before transplanting onto the field.

For each treatment, 10 explants were used. The experiments were carried out thrice and the final results recorded and tabulated after 4 weeks of culturing. The results were expressed as mean±SE using Microsoft Excel.

Histological observation: The vitrified structures (shoot like malformations and tubular embryoids) were fixed in FAA (70% ethanol: formalin: acetic acid), subjected to dehydration through an ethanol-xylol series and embedded in a paraffin wax ($40-60^0$C melting point). The sections (10µm thick), were taken using a microtome, stained with Safranin and mounted on DPX mountant. The sections were observed thoroughly and photographed under a Nikon microscope.

RESULTS AND DISCUSSION

The development of multiple shoots initiated from the ends of nodal and scape explants after 3-5 days of culturing. After 7-10 days in culture, they transformed into elongated, thick, green shoot like structures, 2-4 cm in length, with no visible development of leaves and roots (Figure 1A). Subsequently a number of such structures emerged in clumps from the culture media after 14-16 days leaving no space for development of healthy shoots leading to almost 100% loss of plantlets.

Alternatively, after 6-8 days in culture, the explants produced whitish, tubular embryoid like structures, about 1-2 cm in length. They appeared as a bunch of malformed, abnormal appendages originating from the explants (Figure 1B) and covered the entire surface of the culture media leaving little scope for the emergence of healthy shoots. A noteworthy feature of these vitrified structures was that they had a high pace of growth and formed several such structures in a very short duration of time (6-8 d), primarily affecting the net yield of multiple shoots.

The examination of histological sections of these vitrified structures revealed the globular staged embryo (Figure 1C) and the origination of meristematic structures from the meristemoids (Figure 1D). However the meristem looks disorganized which may be due to dissociation of cell walls due to the failure in their lignification and the absence of definite vascular system and leaf primordia (Rasco *et al.*, 1997).

Since almost 100% loss of plantlets was incurred, we designed a proliferation medium fortified with various adjuvants like coconut water, casein hydrolysate and Phoroglucinol to decrease the incidence of

vitrification. Previous studies on callus culture in *E. foetidum* indicated that BAP had an upperhand in generation and maintenance of organogenic callus.(Ignacimuthu *et al.,* 1999).However it is evident from the results recorded in the present investigation (Table 1), that increased cytokinin concentration (BAP and Kinetin) led to vitrification in the culture medium.

Figure 1: A-E-Vitrification in *E.foetidum*
A-Vitrified shoot like structures in *E. foetidum* rich in chlorophyll; B-Vitrified tubular embryoid structures; C-Histological section indicating globular stage of the somatic embryo; D- Histological section depicting meristematic structure; E,F- Recovery of healthy shoots

Table 1: Effect of cytokinin concentration on multiple shoot culture of *E.foetidum* (Values are mean± SE)

Growth hormones (mg l^{-1})		No. of shoots/explant	Vitrification (%)
BAP	**Kinetin**		
0.5	-	8.4±0.22	24.7
1.0	-	11.8±0.53	27.1
1.5	-	13.3±0.26	42.8
2.0	-	15.4±0.34	63.5
-	0.5	8.1±0.31	23.7
-	1.0	11.4±0.3	27.4
-	1.5	12.4±0.33	47.9
-	2.0	15.1±0.41	65.1

Table 2: Effect of Phloroglucinol, Casein hydrolysate and agar concentration on vitrified shoots after four weeks of culture

Phloroglucinol	Caesin hydrolysate	Agar	Reduction in vitrification (%)
2.0	**5**	**8**	8.3
5.0	**10**	**10**	11.9
10.0	**15**	**12**	13.1

The concentrations of Phloroglucinol, Casein hydrolysate and agar have been expressed in mg l^{-1}.

Hence it was required to keep the cytokinin concentration low to achieve maximum organogenesis and simultaneously reduce vitirification. It was observed that incorporation of coconut water (10% v/v) in the culture medium greatly benefitted rapid callus growth and organogenesis leading to enhanced shoot proliferation which had high survivability. This goes in accordance with the studies in *Citrus* species (Burnet *et al.*, 1973) and *Daucus carota* (Caplin *et al.*, 1948) and possibly be attributed to its auxin and cytokinin like activity (Kurashi *et al.*, 1961; Letham, 1982).

The other adjuvant which proved its efficacy in improving the shoot multiplication was casein hydrolysate. We witnessed a gradual decrease in the levels of vitrification as the concentration of casein hydrolysate was incremented from 5-15 mg l^{-1}(Table 2). However further increase in its concentration had little effect in improving the rate of shoot multiplication. Hence fortification of the culture medium with15 mg l^{-1} of casein hydrolysate was found to be ideal to ease out the path for development of healthy shoots. Previous studies have indicated the usefulness of this ingredient in increasing shoot multiplication and accelerating the growth rate and formation of embryogenic calli and somatic embryos (Ying-Ning, 2010; Khierallah *et al.*, 2013).

The occurrence of *in vitro* shoot vitrification was also controlled with the use of Phloroglucinol (a phenol derivative), in the culture medium. Its inclusion in the culture medium along with casein hydrolysate led to considerable decrease in the formation of vitrified structures. The optimum concentration which supported maximum shoot proliferation was found to be 10 mg l^{-1}. (Table 2). There are several reports suggesting the role of phloroglucinol in proliferation of *in vitro* axillary shoots (Oliveria *et al.*, 2010). It has also been reported that in combination with BAP, phloroglucinol could be used to reduce vitrifications and stimulate proliferation in *in vitro* micropropagation cultures (Gaspar, 1991; Aklan *et al.*, 1997; Salekjalali, 2012). One of the possible ways by which phloroglucinol assists in reducing vitrification is by acting as a precursor in the lignin biosynthetic pathway thus providing the shoots with a rigid framework (Teixeira *et al.*, 2013).

The concentration of the gelling agent, agar agar, plays a critical role in reducing hyperhydricity *in vitro* (Arnold *et al.*, 1984) by increasing the matric potential of the culture medium (Sharma *et al.*, 2006). In view of this, the quantity of agar used in the present investigation was varied from 8-12 mg l^{-1}(Table 2). The medium with highest water stress (12 mg l^{-1}) reduced vitrfication in *E.foetidum* cultures by 13.1%

(Table 2).The increased concentration of the gelling agent might have decreased the water potential of the medium and thereby resulted in improved proliferation of shoots (Muthappa *et al.,* 1998). Nevertheless further increase in the concentration of agar was found to be detrimental for shoot growth and rooting as documented in carnation and *Picea albies* (Arnold *et al.,* 1984). The culture vessels with autoclavable screw capped lids helped in maintaining adequate relative humidity and reducing vitrifications to a great extent (Wardle *et al.,* 1983).

The healthy shoots retrieved from the culture medium incorporated with coconut water, casein hydrolysate, phloroglucinol were normal and devoid of hyperhydricity (Figure 1 E&F). They exhibited good rooting in the same medium and completed their development in about 4 weeks as observed in our earlier studies (Chandrika *et al.,* 2011).

Conclusion

It can be inferred from the above study that vitrifications in *E.foetidum in vitro* cultures can be reduced by fortifying the culture medium with coconut water, casein hydrolysate, phloroglucinol at requisite concentrations. An increase in the matric potential of the culture medium and maintenance of relative humidity in the culture vessel also contributed towards a decline in the percentage of vitrification. This study is vital for the development of an efficient *in vitro* protocol to generate elite clones of the plant for various markets and guard the natural, wild and endemic populations of *E. foetidum*- a potential aromatic and medicinal herb of India.

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