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RESEARCH ARTICLE

Plant Tissue Culture

Induction of somatic embryogenesis from leaf explants of *Exacum trinervium* (L.) Druce (Binara)

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Abstract: *Exacum trinervium,* commonly known as *Binara*, produces dark blue violet flowers with contrasting bright yellow anthers that have a very high potential as commercial flowering, potted, or bedding plants. Lack of planting materials is one of the major constraints for popularizing it and therefore, development of planting materials through *in vitro* somatic embryogenesis would be a viable solution. The possibility of producing somatic embryos of *E. trinervium* using *in vitro* grown leaf explants was investigated. The effect of five concentrations (0, 1, 2, 3 and 5 mg L^{-1}) of 2, 4 - Dichlorophenoxyacetic acid (2,4-D) and 1 - Naphthaleneacetic acid (NAA) on somatic embryogenesis was investigated under dark and light with a photoperiod of 16 hours. The highest percentage of leaf explants (72 %) that produced calli was observed when cultured on the medium supplemented with 1 mg/L 2,4-D. Embryogenic stages could be observed only in calli originating from media supplemented with 1 mg/L and 2 mg/L 2,4-D after transferring to MS medium without growth regulators and incubated both in the light and the complete darkness. Leaf explants cultured on media containing NAA produced heavily rooted calli and did not produce any embryos. The highest number of embryos (15 ± 6 per/mL) was observed in the calli originating from the MS medium containing 1 mg/L 2,4-D. Further development of embryos up to plantlet with 60 % regeneration efficiency could be achieved on MS medium without plant growth regulators.

Keywords: *Binara*, *exacum trinervium*, Somatic Embryogenesis

INTRODUCTION

Exacum trinervium (Trimen) Cramer, commonly known as *Binara*, belongs to family Gentianaceae and tribe Exaceae, is an endemic and near threatened species in Sri Lanka (MOE, 2012). The tribe Exaceae consists of 64 species, dispersed around the Indian Ocean Basin, Africa, Madagascar, India, Sri Lanka, and Australia (Yuan *et al.*, 2005). The highest diversity of genus *Exacum* has been reported in India, Sri Lanka and Madagascar (Klackenberg, 1983).

Exacum trinervium, which produces dark blue violet flowers (4 - 7 cm in diameter) with contrasting bright yellow anthers and attractive glossy foliage has a very high potential as commercial flowering, potted, or bedding plant for the floriculture industry (Riseman & Chennareddy, 2004). However, it is not popular as an ornamental plant in Sri Lanka due to lack of planting materials. Even though *E. trinervium* produces a large number of seeds, the low germination percentage, reduction of seed viability, time consumption, and high cost of production hindered them from being used as a planting material (Riseman *et al*., 2006). Furthermore, propagation of *Exacum* by stem cutting is rather slow and time consuming (Elangomathavan *et al.,* 2003). Thus, there is a need for introducing efficient propagation methods to overcome these problems.

Tissue culture methods are effective at producing a large number of disease free plants within a short time period, compared to conventional methods. Several protocols for micropropagation of different *Exacum* species such as *E. travancornicum* (Elangomathavan *et al*., 2003; Kannan *et al*., 2007; Janarthanam *et al*., 2009), *E. affinae* (Torres & Natarell, 1984; Ballal 1990; Ornstrup

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et al., 1993) and *Exacum styre* group (Unda *et al*., 2007) have been reported. Micropropagation protocols existing for commercially available *E. affine* was not effective for *E. trinervium* (Riseman & Chennareddy, 2004). *Exacum* hybrids originating from Sri Lankan taxa showed a high level of genetic variation, with resulting modification of medium composition for different genotypes (Riseman & Chennareddy, 2004). A complete protocol for propagating *E. trinervium* through multiplication of single nodal cuttings, direct and indirect organogenesis from leaf explants has been reported (Tennakoon *et al*., 2015). However, an efficient somatic embryogenesis protocol for the species is yet to be developed.

Somatic embryogenesis is an efficient clonal propagation technique, which can be used for synthetic seed production (Haque & Ghosh, 2014), and as a model to study the initial events of zygotic embryogenesis in higher plants (Méndez-Hernández *et al.*, 2019). Furthermore, somatic embryogenesis is very useful technique in regenerating plants from selected single cells with desirable genetic characters. Thus, development of somatic embryos would pave a path to improve *E. trinervium* through biotechnological approaches such as integration of genes to change the plant architecture to develop it as a potted ornamental plant.

Therefore, in the present study, the production of plantlets through somatic embryogenesis of *E. trinervium* was investigated with the final objective of introducing it into the floriculture industry.

MATERIALS AND METHODS

Immature leaves from *in vitro* cultured *E. trinervium* plants were excised and approximately 1 cm^2 pieces with a portion of the main vein were cultured on full strength Murashige and Skoog (1969) semi solid medium (MS) with 5 concentrations (0, 1, 2, 3 and 5 mg/L) of 2,4-D and NAA. The media were supplemented with 30 g/L sucrose and solidified with 2.0 g/L phytagel. The pH of the media were adjusted to 5.8 before autoclaving. The cultures were incubated at 20 °C in complete dark to induce callus production. Each treatment was replicated 3 times and each replicate consisted of an average of 20 leaf explants cultured on 25 mL culture tubes. Each culture tube contained a single piece of leaf explant (1 cm^2) on 10 mL of the medium. Cultures were observed for 8 weeks and the number of leaf explants producing calli in each treatment were counted at the time of sub culturing.

Eight weeks after establishment, whole leaf explants with initiated calli from different media were separately transferred to full strength MS solid medium supplemented with 2.0 g/L phytagel without any plant growth regulator (PGR). Part of the cultures were incubated continuously at 25 °C in complete dark conditions while other part was transferred to light (1300 lux, 16 h photoperiod). The callus lines were maintained separately based on the composition of the original medium where the callus was initiated, to identify the effect of type and concentration of auxin in the original medium for somatic embryogenesis. Growth of the callus or roots was measured by weighing at the time of transferring under aseptic conditions inside the laminar flow cabinet, using a Mettler Analytical balance (Mettler, Germany). Calli were observed using a dissecting microscope (XT-Series-Alltion) at 2 wks interval for the production of somatic embryos.

Then 16 wks after establishment of cultures, calli originating from MS media supplemented with 1 and 2 mg/L 2,4-D and transferred to MS solid medium without growth regulators were again transferred to full strength MS liquid and solid media without any plant growth regulators. These two callus lines were incubated only in light to identify the effect of liquid medium for separation of somatic embryos. Cultures were observed under a dissecting microscope (XT-Series-Alltion) at 2 wks intervals by taking destructive samples for the production of somatic embryos. The number of embryos present was counted by taking 1 mL of the sample by using a micro pipette (Gilsons - 5 mL) with sterile tips inside the laminar flow cabinet and observing through a dissecting microscope (XT-Series-Alltion). Different stages of somatic embryogenesis were also documented by using photographs.

The initiated somatic embryos were transferred to MS medium without growth regulators for germination and the number of regenerated plantlets was counted. Regenerated plantlets were taken out from culture jars and washed by using warm water to remove adhesive agar and the medium. Then the plants were acclimatized by placing inside culture jars containing 1 cm layers of gravel, coir dust and sand. These jars were sterilized by autoclaving before placing the plantlets under sterile conditions inside a laminar flow cabinet. The plantlets were kept two weeks in culture room prior to transferring into the plant house. Jars were opened gradually to exposed plants to the open environment inside the plant house. Then the acclimatized plantlets were transferred to soil and grown up to flowering in the plant house (Tennakoon *et al.*, 2015).

Data analysis

All the experiments were arranged in completely randomized design. The number of explants producing calli/roots at different concentrations of 2, 4-D and NAA were analyzed using the Chi-Square test. The effect of dark and light on the growth of the callus after transferring to growth regulator free media was analyzed by using ANOVA considering the light condition as one factor and the concentrations of 2, 4-D and NAA present in the medium where calli was originated from leaf explants as the second factor. Numbers of somatic embryos initiated on different media were compared using the Chi-Square test.

RESULTS AND DISCUSSION

Selection of the correct development stage of the explant is important for somatic embryogenesis. Young and fast dividing parts of the plant with least microbial infections are considered as the most favourable explants for embryogenic callus formation (Ornstrup *et al.*, 1993). In the present study somatic embryogenesis of *E. trinervium* was achieved by using immature leaf explants. The risk of contaminations could be minimized and immature leaves could be obtained easily since *in vitro* plants were used as the mother plants. Hamidah *et al.* (1997) also identified leaf pieces obtained from micropropagated plants as the best explant for the induction of somatic embryogenesis of *Anthurium scherianum*.

Calli initiation from the cut margins of the leaf explants (Figure 1a) started 3-4 wks after establishing the cultures on media supplemented with 2,4-D and NAA. Calli initiated from explants cultured on MS media supplemented with 2,4-D gradually became yellowish and further multiplied (Figure 1b and 1c) while the explants producing calli on MS media supplemented with NAA produced white colour roots and rapid multiplication of roots was observed (Figure 1d and 1e). Medium without auxins produced neither calli nor roots (Figure 1f).

Eight weeks after establishment of cultures, the highest percentage (72 %) of explants had produced calli on the medium supplemented with 1 mg/L 2,4 D concentration and it was significantly ($p < 0.05$) higher compared to all the other growth regulator concentrations (Figure 2). However, at this stage calli or roots were difficult to separate from the explants and therefore, whole explants with initiated calli or roots were transferred to MS media without plant growth regulators.

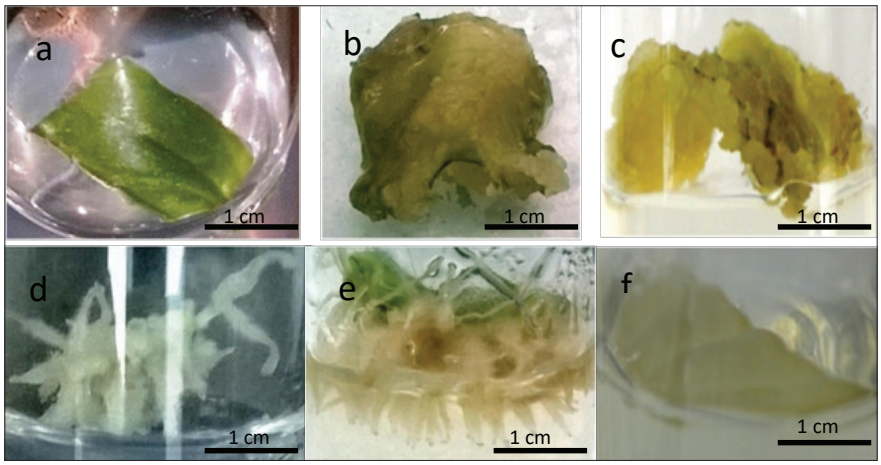

Figure 1: Effect of 2,4-D and NAA on callus/root initiation from leaf explants: (a) Leaf explant at the time of establishment. (b) Soft light yellow callus on edges of leaf explant in 2,4-D media. (c) Multiplication of callus initiated on 1 mg/L 2,4-D after transferring to growth regulator free MS. (d) Root initiated callus in NAA media. (e) Multiplication of white colour rooted callus initiated on 1 mg/L NAA after transferring to growth regulator free MS medium in the dark. (f) Leaf explant cultured in the dark on medium without auxin.

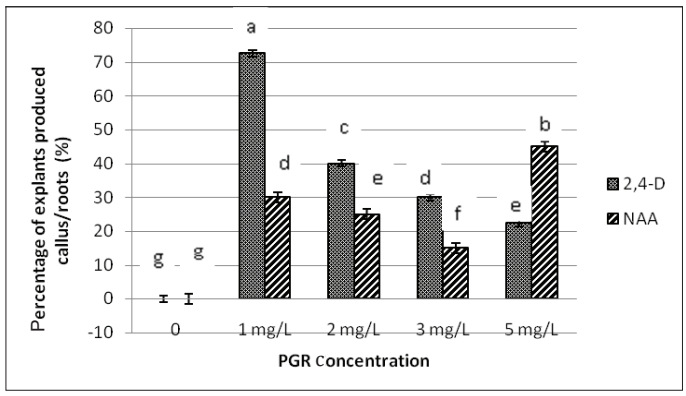

Figure 2: Production of callus on immature leaf explants after eight weeks from the establishment of cultures on MS media supplemented with different concentrations of 2,4 –D and NAA, (Note: Bars indicated by same letters are not significantly different)

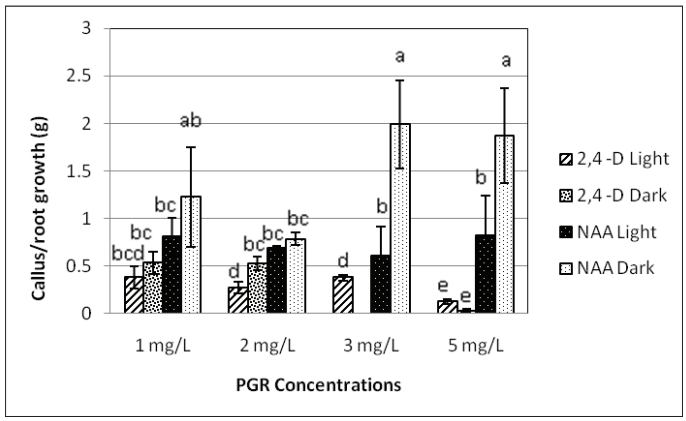

Figure 3: Effect of light conditions, 2,4-D and NAA on callus/root weight (Note: Bars indicated by same letters are not significantly different)

Calli initiated on media supplemented with 2,4-D produced more calli under both conditions, the complete darkness and the light (16 h light / 8 h dark at 1300 lux) when they were transferred to MS medium without growth regulators. Furthermore, calli incubated in the dark produced yellowish, friable calli (Figure 1c) while yellowish green colour friable calli were produced by cultures incubated under light conditions. Calli originated from MS medium supplemented with NAA produced more roots under both light and dark conditions, after transferring to MS medium without growth regulators (Figure 1e).

After 16 weeks of establishment (8 weeks after transferring to growth regulator free medium) calli/root weight were taken to determine the effect of growth regulators (2,4-D/NAA) present in the original medium and light on calli/root growth. A significant difference ($\alpha = 0.05$) could be observed between dark and light conditions and between composition of 2,4-D and NAA in the callus initiating medium on further growth of callus and roots after transferring to MS medium without growth regulators (Figure 3). MS media supplemented with NAA have shown a higher weight increase compared to MS media supplemented with 2,4-D and this was mainly due to rapid root production (Figure 3). Furthermore, significant increase in callus/root growth was observed when the cultures were maintained under dark conditions irrespective of the type of the growth regulators.

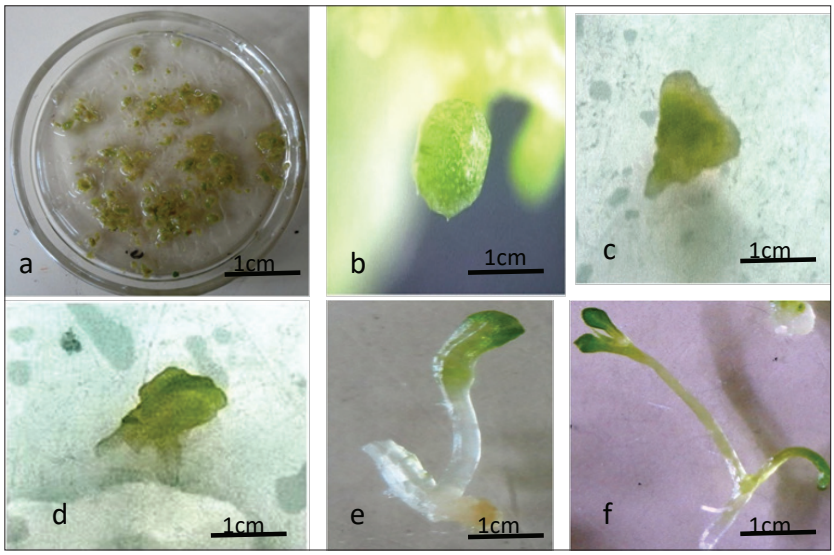

Figure 4: Different embryogenic stages in liquid MS medium: (a) Development of somatic embryos in liquid medium-[x1(normal eye)] (different stages of somatic embryos isolated from the same culture observed using a dissecting microscope are shown in Figure 4b-f). (b) Globular stage (x40). (c) Heart stage (x40). (d) Torpedo stage (x40). (e) Torpedo stage with root initials (x40). (f) Plantlets [x1(normal eye)] at 20 weeks of establishment.

Two weeks and four weeks after transferring (10 and 12 weeks after establishment) to plant growth regulator free MS media, different embryogenic stages could be observed in calli which were initiated in MS media with 1 mg/L and 2 mg/L 2,4-D concentrations and incubated under both light and dark conditions (Figure 4). However no somatic embryogenesis was observed from a callus originating from any explants cultured on MS media containing NAA. The role of growth regulators is very important for cultured cells and tissues in developing a specific mode of growth. Auxins such as NAA promote *in vitro* plant rooting (Ngomuo *et al.*, 2013). Furthermore, Lazzaeri *et al.* (1987) have reported that when similar concentrations of 2,4-D and NAA were compared, 2,4-D produced a larger number of somatic embryos in soybean. Four weeks after transferring (12 wks after establishment) of calli to MS media without growth regulators, further development of embryogenic stages could be observed.

Two weeks after transferring to liquid medium, different developmental stages of somatic embryos could be easily identified since they were well separated from each other in the liquid medium. A higher number of somatic embryos (15 ± 6 per mL) were produced by calli originated on MS solid medium supplemented with 1 mg/L 2,4 D compared to that of 2 mg/L 2,4-D) (10 ± 5 per mL). However, results were not significantly different. Four weeks after transferring to liquid medium (20 weeks after establishment) development of plantlets could be observed (Figure 4).

Auxin plays a major role in induction of embryogenesis in cultures and further development of embryos (Li *et al*., 1999; Nurazah *et al*., 2009). In the initial media, tissues get the ability to synthesize all the gene products required to complete the globular stage of embryogenesis (Komamine *et al.*, 1992). Once embryogenesis has been induced embryos start to produce endogenous auxin and the role of auxin changes (Fan *et al.*, 2012). The cultures have to be transferred to a medium with reduced or free of auxin (Zimmerman, 1993). In accordance with that in the present study the calli that were induced in the initial MS medium supplemented with auxin were transferred to full strength MS medium without growth regulators 8 weeks after establishment to induce embryogenic ability. However Gawel *et al.* (1986) have transferred the calli initiated on leaf disc explants of *Gossypium hirsutum* L. two weeks after establishment. Furthermore, calli initiated on leaf explants of *Anthurium scherzerianum* have been transferred 4 weeks after establishment (Hamidah *et al.*, 1997). This implies that the time of transferring of calli to auxin free media varies with the plant species.

In this study the transferring time was decided according to the callus formation percentage. The transferring was done when the majority (75%) of the explants produced calli. Transferring time should be carefully decided since a longer or shorter time period of cultures in initial media may result in the loss of the embryogenic ability of the cultures (Nurul *et al.*, 2016)

According to the results of the present study 2,4-D was the most effective plant growth regulator on induction of somatic embryogenesis of *E. trinervium*. Although explants in the initial MS medium supplemented with different concentrations of 2,4-D and NAA produced different percentages of calli, only 1 mg/L and 2 mg/L 2,4-D was effective in producing embryogenic calli from immature leaf explants. The presence of higher concentrations of 2,4-D has showed an inhibitory effect on callus formation of *E. trinervium* (Tennakoon *et al.*, 2015). Ornstrup *et al.* (1993) have reported that 2,4-D was essential in achieving somatic embryogenesis of *Exacum affine.*

Somatic embryogenesis is a useful method especially as a solution for issues related to plant breeding such as problems with floral morphology, sterility, and time consumption. This is also useful as a method of genetic improvement of plant species and multiplication of valuable germplasm. In the case of *E. trinervium,* somatic embryogenesis can be used to overcome problems regarding commercialization of the plant.

CONCLUSION

Somatic embryogenesis of *E. trinervium* could be achieved by culturing immature leaf explants on MS media supplemented with 1 mg/L 2,4-D, under both conditions, total darkness and light, followed by transferring to solid MS medium without plant growth regulators, and finally transferring to liquid MS medium without growth regulators.

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