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In vitro Plant Regeneration from Diploid and Tetraploid *Exacum ritigalensis* (Binara)

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ABSTRACT

Purpose: *Exacum ritigalensis (Binara/Ginihiriya) is an endemic plant found in Sri Lanka which has valuable ornamental characteristics. A protocol is timely important to develop for rapid, efficient multiple callus induction, shoot induction and rooting. Stem and leaf explants on Murashige and Skoog (MS) medium supplemented with different plant growth regulators were used for the study. The aim of the present study was to encompass the effects of plant growth regulators and explants on in vitro callus production, shoot regeneration and root formation of Exacum ritigalensis in diploid (28 n) and tetraploid (56 n) plants which have been exploited for genetic improvement programmes.*

Research Method: *MS basal medium with 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, mgL^{-1} 6-Benzylaminopurine (BAP) with 0.1mgL^{-1} 2,4-Dichlorophenoxy acitic acid (2,4-D) were used to produce callus from aseptically produced stem cuttings and leaves. MS basal medium with 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, mgL^{-1} BAP and 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, mgL^{-1} Indole-3-butyric acid (IBA) were used for shoot and root induction respectively. Complete Randomized Design (CRD) with 10 replicates was used for each treatment and repeated the experiments five times.*

Findings: *Results showed that stem cuttings were the best for callus induction compared to leaf explants of both diploid (1.4±0.1 cm^2) and tetraploid (1.0±0.2 cm^2) plant of E. ritigalensis within 19±2 days in MS basal medium supplemented with 2.0 mgL^{-1} BAP with 0.1 mgL^{-1} 2,4-D. The best mean number of shoot proliferation from callus (diploid = 16.0±1.0 and tetraploid = 11.5±0.5) and stem cuttings (diploid = 12.0±2.0 and tetraploid = 10.5±0.5) were observed on MS basal medium supplemented with 2 mgL^{-1} BAP with the highest shoot length. The highest number of roots (diploid =11.5±0.1 and tetraploid = 9.5±0.2) and root length (diploid = 3.2±1.7 cm and tetraploid = 2.2±0.3 cm) were observed on MS medium with 2 mgL^{-1} IBA.*

Research Limitations: *Cytokinin (BAP) was used for induce both callus and shoot regeneration.*

Originality/value: *The present study was developed a protocol for regeneration of diploid and tetraploid plants of E. ritigalensis.*

Keywords: *6-Benzylaminopurine, Callus, 2,4-Dichlorophenoxy acitic acid, Exacum ritigalensis, Indole-3-butyric acid*

INTRODUCTION

Exacum ritigalensis (Binara) is an indigenous wild plant that belongs to the family Gentianaceae and tribe Exaceae. Together with 64 species are dispersed around the Indian Ocean Basin, Africa, Madagascar, Socotra, the Arabian Peninsula, Sri Lanka, India, the Himalayas, mainland Southeast Asia including southern China, Malaysia, and northern Australia (Yuan *et al.*, 2005; Sumanasinghe, 2012). This is an

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annual erect herb 50 – 100 cm in height which produces light blue to dark blue hermaphrodite flowers with contrasting bright yellow anthers and with shiny green foliage. It has a four sided slender stem with upright branching and is found in moist soil in forest areas. *Exacum* plant is desirable for horticultural applications because of the beautiful appearance with blue colored flowers. Chromosome number of *Exacum ritigalensis,* diploid plant is 28 and tetraploid is 56 (Perera *et al.,* unpublished).

Binara is distributed in the intermediate wet zone forests of Sri Lanka and it can be found in Kurunegala, Anuradhapura, Kandy and Badulla districts (Trimen *et al.,* 1931). Six *Exacum* species from Sri Lanka have been evaluated for horticultural merits in USA and those accessions have shown potential to be improved as pot plants, bedding plants and for cut flower production (Riseman and craig, 1995). Naturally *E. ritigalensis* is propagated by seeds. Even though large numbers of very small seeds (150-250) are produced within a capsule, germination percentage is less than 10% (20-25 seedlings per capsule) (Riseman *et al.*, 2006). Conservation of endemic *Exacum* species is concerned because they are not widespread and may be confined only to one or two protected areas. *Exacum* replanting in natural habitat is not easy because they are adapted only to special environmental conditions and seed of tetraploid plants exists with poor germination ability. Thus, the present study was conducted to develop a simple, rapid, economical, and high frequency micro-propagation protocol for diploid and tetraploid plants of *E. ritigalensis,* to conserve the species as well as introducing it as a potted ornamental plant in floriculture industry.

MATERIALS AND METHODS

Establishment of aseptic cultures:

Pods of *Exacum* were collected from forest in Pannala, Kurunegala district, Sri Lanka. Pods of diploid plants were surface-sterilized by washing under running tap water (1/2 hour) and soapy water (15 minutes). Pods were then immersed in 70% ethanol for 3 minutes and rinsed three times with distilled water. Then pods were disinfected with 20% Clorox (Sodium hypochloride) for 20 minutes in the laminar air flow cabinet. Sterilized pods were then rinsed three times with sterilized distilled water. They were dried onto sterile filter papers. Pod coat was removed with sterile scalpels and pliers. The seed culture medium was prepared with only using 0.8% Agar with a pH of the medium adjusted to 5.8-6.0 with 1N NaOH or 1N HCl solution prior to autoclaving at 1.5 $kgcm^{-2}$ for 20 minutes. The ten seeds were transferred into the prepared medium in 250 ml bottles and cultured in culture room under 25±2°C having 16 h/8 h photoperiod under cool white fluorescent light with 1000-1300 Lux intensity conditioned room for two months. For the experiment, explants of diploid plants were taken from *in vitro* tissue culture plants and tetraploid plants were taken from *in vitro* cochicine treated tissue culture plants (Perera *et al.,* unpublished).

Effect of type of explants, BAP and 2,4-D on callus production in diploid and tetraploid plants

Two months old leaf and stem explants from aseptic plantlets were cultured on MS basal medium (Murashige and Skoog, 1962) with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mgL^{-1} BAP with 0.1 mgL^{-1} 2,4-D, and hormone free medium (pH-5.8) to initiate the callus (Inoka *et al.,* 2014). In cultures, leaves were cut into sections (0.5 cm^2) and placed on media with the adaxial surface touching the medium. Single node stems were cut into 0.5 cm pieces before introducing to the above media. Then callus induction time was evaluated and after one month of culture, area of callus (cm^2) was measured.

Effects of BAP on shoot proliferation from single node stem cuttings

The single nodal stem cuttings (0.5 cm) from aseptic plantlets were established on MS medium (pH 5.8) at six BAP concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mgL^{-1}) and

hormone free medium (control). One month after establishment, the number of shoots produced per node and height of shoots were recorded and analyzed.

Effect of BAP on shoot regeneration from callus

The calluses (0.5cm^2) from stem cutting explants were established on MS medium (pH 5.8) at six BAP concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mgL^{-1}) and control treatment (without hormones) were used. After one month, the number of shoots produced per callus and height of shoots were recorded and analyzed.

Effect of IBA on rooting of develop shoots from single node stem cuttings

In vitro multiplied shoots were established on MS medium containing six different concentrations of IBA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mgL^{-1}) and IBA free medium. Number of roots per shoot and average root lengths were recorded at one month after transferring to the above medium.

In all the experiments, the cultures were incubated at 25±2°C with 16 h/8 h photoperiod under cool white fluorescent light with 1000-1300 Lux.

Experiment design and Statistical analysis

Experiment was arranged according to the Complete Randomized Design (CRD). All experiments had repeated three times, each with ten replicates. Statistical analysis was carried out using Duncan's multiple range test at 5% level of SAS program (9.1.3).

RESULT AND DISCUSSION

Effect of explants and BAP and 2,4-D on callus production

In the present study, stem and leaf explants of *E. ritigalensis* were cultured on MS basal medium supplemented with various concentrations and combinations of BAP with 2,4-D for the induction of callus (Table 01). *Exacum* tissue culture protocol is different from other typical tissue culture protocols for callus induction. It needs MS medium with high level of cytokinin with low concentration of auxin (Inoka *et al.,* 2014). In both stem and leaf explants, callus initiation was significantly the highest (P≤0.05) in MS basal medium containing 2 mgL^{-1} BAP and 0.1 mgL^{-1}2,4-D (Figure 01, A and Figure 02, A) for diploid and tetraploid plants. Callus inductions of stem explants were shorter (10-15 days) compared to leaf explants (15-20 days). Inoka *et al.,* (2014) showed that the time taken to callus induction was 10-15 days from stem cuttings of *E. sessile*. However, in this experiment, there was no significant difference between the time which had taken to initiate callus in both stem and leaf explants. Thimmayan *et al.,* (2011) and Inoka *et al.,* (2014) observed the highest callus production (1.4±0.5 cm^2) from MS basal medium with 2.0 BAP + 0.03 NAA mgL^{-1}. Alike as in this experiment Yunfei *et al.* (2012) demonstrated that 2,4-D is efficient for callus induction in *Gentiana straminea* Maxim, a member of the Gentianaceae family. The callus formation of *Exacum bicolor* Roxb was more pronounced (80.03 %) in the MS basal medium supplemented with BAP and 2,4-D at 1.5 and 0.9 mg/L^{-1} respectively (Meethaley and Subramaniam, 2011). Callus production from stem cuttings was higher than from leaves in both diploid and tetraploid plants. Similar results were observed from Unda *et al.,* (2007) where callus from leaf explants of *Exacum* Styer group supplemented with 1 or 2 mgL^{-1} BAP in combination with low concentrations of 0.01 or 0.5 mgL^{-1} NAA. It has been reported that the position of the leaf from where explants are excised could determine the *in vitro* response and leaf sections obtained from the basal parts are the best (Haydu and Vasil, 1981; Tennakoon *et al.,* 2015).

Table 01: Effect of different BAP and 2,4-D hormone combination for callus induction from leaf an stem explants in diploid and tetraploid plant

Different BAP concentrations mgL^{-1} + 0.1mgL^{-1} 2,4-D	Mean area of callus from stem explants (cm^2) (mean±SD)		Time taken to initiated callus (days) from stem (mean±SD)		Mean diameter of callus from leaf explants (cm^2) (mean±SD)		Time taken to initiated callus (days) from leaf (mean±SD)	
	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid
0	0.0±0.0^{e}	0.0±0.0^{e}	-	-	0.0±0.0^{d}	0.0±0.0^{c}	-	-
0.5	0.3±0.1^{d}	0.2±0.1^{d}	27±2^{a}	27±2^{a}	0.0±0.0^{d}	0.0±0.0^{c}	-	-
1	0.7±0.1^{c}	0.6±0.1^{c}	27±2^{a}	27±2^{a}	0.3±0.2^{c}	0.0±0.0^{c}	22±2^{b}	-
1.5	1.0±0.2^{b}	0.8±0.2^{b}	20±2^{c}	21±2^{b}	0.8±0.1^{b}	0.4±0.1^{b}	22±2^{b}	24±2^{b}
2	1.4±0.1^{a}	1.0±0.2^{a}	19±2^{c}	21±2^{b}	1.2±0.2^{a}	0.5±0.1^{a}	20±2^{c}	21±2^{c}
2.5	1.1±0.1^{b}	0.8±0.1^{b}	19±2^{c}	22±2^{b}	0.9±0.1^{b}	0.5±0.1^{a}	27±2^{a}	27±2^{a}
3	0.3±0.1^{d}	0.1±0.2^{ed}	24±2b	27±2^{a}	0.0±0.0^{d}	0.0±0.0^{c}	-	-

**Means followed by the same lower case letters in each column are not significantly different at 5% level in Duncan's Multiple Range Test*

Effect of BAP on shoot proliferation using single node stem cuttings

All the stem cutting explants responded to all BAP tested, (exempt 0 mgL^{-1}) concentrations by shoot formation (Table 02). Results showed that proliferation of shoots was the most efficient in the MS medium supplemented with 2 mg L^{-1} BAP. Lower shoot height was observed in tetraploid (0.93±0.2 cm) plants in comparison with diploid plants (5.20±0.3 cm) (Figure 01, B and Figure 02, B). In contrast to the results, the highest proliferation of shoots was obtained with MS medium containing 2 mgL^{-1} BAP for *Exacum trinervium* and MS medium with 1 mgL^{-1} BAP for *Centaurium erythraea* (Family-Gentianaceae) (Tennakoon *et al.,* 2015; Ewelina *et al.,* 2011). The results of Rodica *et al*., 2016 showed that the medium with BAP was the most effective for obtaining the highest shoots from *Lisianthus russelianus*.

Table 02: Effect of different BAP concentrations for shoot induction from single node stem cuttings in diploid and tetraploid plants

BAP concentrations (mgL^{-1})	Mean number of shoots per cutting (mean±SD)		Mean height of shoot (cm) (mean±SD)	
	Diploid	Tetraploid	Diploid	Tetraploid
0	0.0±0.0^{f}	0.0±0.0^{e}	0.00±0.0^{e}	0.00±0.0^{f}
0.5	5.0±1.0^{e}	2.0±0.5^{d}	0.70±0.1^{d}	0.20±0.2^{e}
1	7.0±0.5^{d}	6.0±1.0^{c}	3.10±0.1^{c}	0.40±0.2^{d}
1.5	8.5±1.0^{c}	8.0±0.5^{b}	3.30±0.1^{b}	0.50±0.2^{cd}
2	12.0±2.0^{a}	10.5±0.5^{a}	5.20±0.3^{a}	0.93±0.2^{a}
2.5	10.5±1.0^{b}	7.5±0.5^{b}	0.80±0.2^{d}	0.70±0.1^{b}
3	8.0±1.0d^{c}	7.5±0.5^{b}	0.73±0.3^{d}	0.60±0.1^{cb}

**Means fallowed by the same lower case letters in each column are not significantly different at 5% level in Duncan's Multiple Range Test.*

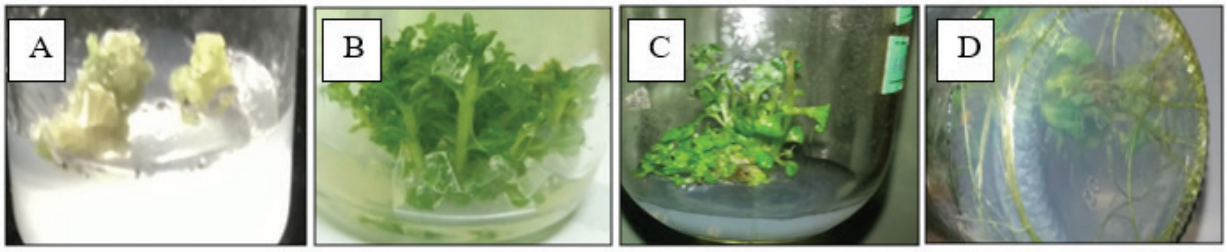

Figure 01: **Diploid plant callus production and shoot and root regeneration after 30 days. A, callus induction from the stem explants (2.0 mgL^{-1} BAP with 0.1 mgL^{-1} 2,4-D); B, shoot proliferation from stem cutting (2.0 mgL^{-1} BAP) ; C, shoot proliferation from callus (2.0 mgL^{-1} BAP) and D, rooting (2.0 mgL^{-1} IBA).**

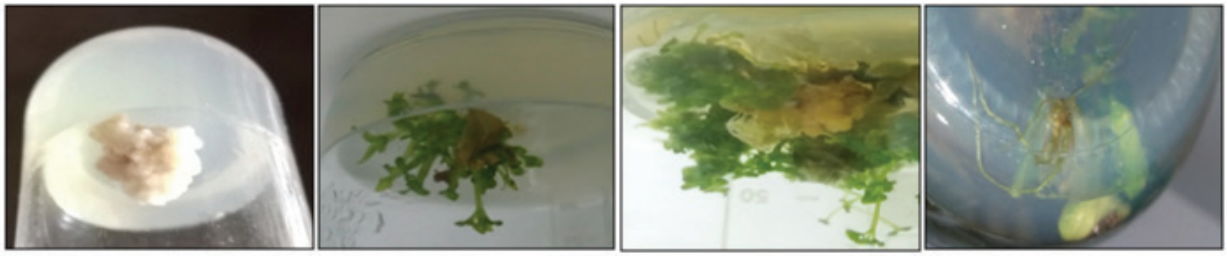

Figure 02: **Tetraploid plant callus production and shoot and root regeneration after 30 days. A, callus induction from the stem explants (2.0 mgL^{-1} BAP with 0.1 mgL^{-1} 2,4-D); B, shoot proliferation from stem cutting (2.0 mgL^{-1} BAP) ; C, shoot proliferation from callus (2.0 mgL^{-1} BAP) and D, rooting (2.0 mgL^{-1} IBA).**

Effect of BAP on shoot regeneration from callus of E. ritigalensis

BAP induced shoots in single node stem cuttings (Table 02) as well as it was induced callus production of *E. ritigalensis* (Table 03). The best shoot regeneration in both diploid and tetraploid callus was showed in the media supplemented with 2 mgL^{-1} BAP (Figure 01, C and Figure 02, C). The callus produced a higher number of buds (16±1) than stem cuttings (12±2). Similar results were observed when sub-culturing the callus of *Exacum bicolor* Roxb for shoot formation in the medium containing BAP and NAA at 1.0 and 0.2 mg L^{-1} respectively (Meethaley and Subramaniam, 2011).

Table 03: **Effect of different hormone combinations for shoot induction from callus in diploid and tetraploid plants**

BAP concentrations (mgL^{-1})	Mean number of shoots per callus (mean±SD)		Mean height of shoots (cm) (mean±SD)	
	Diploid	Tetraploid	Diploid	Tetraploid
0	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{d}
0.5	0.3±0.5^{e}	0.5±0.5^{e}	0.5±0.1^{d}	0.2±0.1^{d}
1	10.5±0.5^{d}	8.0±1.0^{c}	2.5±0.6^{c}	0.5±0.2^{c}
1.5	11.5±0.5^{c}	10.0±0.5^{b}	3.5±0.4^{b}	0.6±0.2^{cb}
2	16.0±1.0^{a}	11.5±0.5^{a}	4.2±0.7^{a}	1.2±0.4^{a}
2.5	14.5±0.5^{b}	8.5±0.5^{c}	0.8±0.5^{d}	0.8±0.2^{b}
3	14.0±0.5^{b}	7.0±0.5^{d}	0.8±0.4^{d}	0.8±0.1^{b}

**Means fallowed by the same lower case letters in each column are not significantly different at 5% level in Duncan's Multiple Range Test*

Table 04: Effect of different IBA hormone concentrations for root induction in diploid and tetraploid plants from stem cuttings

IBA concentrations (mgL^{-1})	Mean number of roots per shoot (mean±SD)		Root length cm (mean±SD)	
	Diploid	Tetraploid	Diploid	Tetraploid
0	00.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{d}	0.0±0.0^{d}
0.5	04.0±0.1^{d}	0.0±0.0^{e}	0.7±0.4^{d}	0.0±0.0^{d}
1	07.5±0.3^{c}	6.1±0.3^{e}	1.5±0.2^{c}	1.3±0.2^{b}
1.5	10.0±0.1^{ab}	7.0±0.2^{d}	2.5±0.4^{b}	1.4±0.4^{b}
2	11.5±0.1^{a}	9.5±0.2^{a}	3.2±1.7^{a}	2.2±0.3^{a}
2.5	08.8±0.2^{bc}	8.3±0.1^{b}	1.8±0.6^{bc}	0.8±0.4^{c}
3	08.5±0.2^{bc}	8.0±0.1^{c}	1.8±0.4^{bc}	0.7±0.5^{c}

**Means fallowed by the same lower case letters in each column are not significantly different at 5% level in Duncan's Multiple Range Test*

Effect of IBA on rooting of* E. ritigalensis *shoots

Except control treatment, diploid shoots of *E. ritigalensis* were responded to all other IBA concentrations used in this study. However, the IBA concentration of 0.5 mgL^{-1} did not promote the roots in tetraploid plant. Low concentrations of IBA showed reduced root formation in this experiment (Table 04) and it was quoted from Tennakoon *et al.,* (2015) that the low concentrations of IBA were not effective for rooting of *E. trinervium.* The highest number of root and root length was shown in IBA concentration of 2 mgL^{-1} in both diploid and tetraploid plant (Table 04). The present results were justified from Meethaley and Subramaniam (2011) for *Exacum bicolor* Roxb, Rodica *et al.,* (2016) for *Lisianthus russelianus* and Tennakoon *et al.,* (2015) for *E. trinervium.*

CONCLUSION

Results of the present study revealed that the single node stem cutting (0.5 cm) explants were found to be the best for callus induction comparing to leaf and root explants of diploid and tetraploid *E. ritigalensis*. The best callus production was observed within 19±2 days on MS basal medium supplemented with 2.0 mgL^{-1} BAP with 0.1 mgL^{-1} 2,4-D. Diploid and tetraploid plants of *E. ritigalensis* shoot regeneration were observed from both shoot cuttings and callus of MS medium with 2.0 mgL^{-1} BAP. Root initiation was achieved from shoots/buds through direct organogenesis using 2.0 mgL^{-1} IBA.

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REFERENCES

Ewelina, P., Aleksandra, K., and Halina, W. (2011). Morphology, secoiridoid content and rapid analysis of plants regenerated from callus of *Centaurium erythraea* Rafn. Acta Biologica Cracoviensia Series Botanica. 53(2): 79-86. DOI: http://dx.doi.org/10.2478/v10182-011-0030-3

Haydu, Z., and Vasil, I.K. (1981). Somatic embryogenesis and plant regeneration from leaf tissues and anthers of *Pennisetum purpureum* Schum. Theoritical and Applied Genetics. 59: 269-273. DOI: http://dx.doi.org/10.1007/BF00264978. PubMed. 08.08.2017.

Inoka, K.P.I., Dahanayake, N. and Perera, P.C.D. (2014). Effect of explants and hormone levels on callus production of Binara (*Exacum* spp.). National Symposium on Floriculture Research: 15-18. DOI: http://dx.doi.org/10.21921/jas.v3i4.6712

Meethaley, V.J. and Subramaniam, P. (2011). Evaluation of certain flavonoids of medicinal importance in the wild and micropropagated plants of the endangered medicinal species, *Exacum bicolor* Roxb. Journal of Applied Pharmaceutical Science. 1(05): 99-102. DOI: http://dx.doi.org/10.1007/978-0-387-70638-2_611

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology of Plant. 15: 473-479. DOI: http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Perera, P.C.D., Dahanayake, N., Senanayake, S.G.J.N. and Krishnarajah, S.A. (unpublished). Conservation of Binara (*Exacum ritigalensis*) and enhancement of its floral characteristics through chromosome doubling. M.Phil. thesis (Sri Lanka: Faculty of Post Graduate, University of Ruhuna). DOI: http://dx.doi.org/10.21921/jas.v3i4.6712

Riseman, A., Sumanasinghe, V.A., and Craig, R. (2006). Cytology, crossability, and pollen fertility of Sri Lankan *Exacum* (Gentianaceae) and their hybrids. Journal of Plant Science. 167: 191–199. DOI: http://dx.doi.org/10.1086/499610

Riseman, A. and Craig, R. (1995). Inter specific *Exacum* hybrids: Novel germplasm for the production of a new floriculture crop. Acta Horticulturae. 420:132–134. DOI: http://dx.doi.org/10.17660/actahortic.1995.420.36

Rodica, P., Maria C., Erzsebet B. and Iudita C. (2016). *In vitro* plant propagation and crop improvement in Lisianthus (*Lisianthus russelianus* Hook.), Bulletin UASVM Horticulture. 73(2): 168-171. DOI: http://dx.doi.org/10.15835/buasvmcn-hort:12367

Sumanasinghe, V.A., Karunasena, K.D.I.P., and Wijesundera, D.S.A. (2012). Molecular characterization of selected Sri Lankan *Exacum* Species, International Conference on Agriculture, Chemical and Environmental Sciences: 6-7. DOI: http://dx.doi.org/10.1086/499610

Tennakoon, S., Peiris, S.E. and Eeawara, J.P. (2015). *In vitro* regeneration of *Exacum trinervium,* Acta Horticulturae: 237-249. DOI: http://dx.doi.org/10.17660/actahortic.2015.1104.36

Thimmayan, B., Karuppannan, A., Subramaniam, P., Madathupatti, R.U. and Uthaman, D. (2011). *In Vitro* regeneration of *Exacum wightianum* Arn. (Gentianaceae) - An Endemic Medicinal Plant from the Nilgiris, Western Ghats, Journal of Applied Pharmaceutical Science 1(10): 167-171. DOI: http://dx.doi.org/10.1201/9781315366241-9

Trimen, H., Hooker, J.D. and Alston H.G.A. (1931). Flora of Ceylon, Volume 09, London, Dulau and Company: 54-69. DOI: http://dx.doi.org/10.5962/bhl.title.10864

Unda, F., Kalynyak, P., and Riseman, A. (2007). Organogenesis plant regeneration from leaf explants of *Exacum Styer* group, Plant Cell Tissue culture. 89: 105-111. DOI: http://dx.doi.org/10.1007/s11240-007-9218-3

Yuan, Y.M., Wohlhauser, S., Möller, M., Klackenberg, J., Callmander, M. and Küpfer, P. (2005). Phylogeny and biogeography of *Exacum* (Gentianaceae): a disjunctive distribution in the Indian Ocean basin resulted from long distance dispersal and extensive radiation, System Biology. 54: 21–34. DOI: http://dx.doi.org/10.1080/10635150590905867.PubMed. 14.05.2017.

Yunfei, C., Yan, L.L., Yanfei, W., Fengning X. and Guangmin X. (2012). High frequency somatic embryogenesis and plant regeneration with high content of gentiopicroside from the Chinese medicinal plant, *Gentiana straminea* Maxim. http://www.paper.edu.cn. 12.08.2017. DOI: http://dx.doi.org/10.1007/s11627-009-9225-7