Pak. J. Bot., 48(2): 561-566, 2016.

THE CALLUGENIC EFFECTS OF 2,4-DICHLOROPHENOXY ACETIC ACID (2,4-D) ON LEAF EXPLANTS OF SABAH SNAKE GRASS (*CLINACANTHUS NUTANS*)

QIAN YI PHUA, CHEE KEONG CHIN, ZIYAD RAFIQI MOHAMMAD ASRI, DANNY YUE AUN LAM, SREERAMANAN SUBRAMANIAM AND BEE LYNN CHEW*

School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia
**Corresponding author's email: beelynnchew@usm.my*

Abstract

Clinacanthus nutans (Burm. f.) *Lindau* is a medicinal plant that is widely used in traditional treatments for skin inflammation, skin lesions, viral infection and snake bites. This study investigates the effects of different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) on the induction of callus from *Clinacanthus nutans* leaves. Young and mature leaves of *Clinacanthus nutans* were placed on MS media supplemented with ten concentrations of 2,4-D (0 to 10mg/L). The percentage of callus formation, callus fresh weight and callus morphology were recorded followed by histological analysis on callus from young leaves and mature leaves. The highest mean of fresh weight of callus was obtained from young leaf explants in MS media supplemented with 0.5 mg/L 2,4-D whereas the highest mean of callus fresh weight from mature leaf explants was achieved in MS media supplemented with 0.25 mg/L 2,4-D. Histological analysis indicated potential formation of somatic embryos from callus of young leaf explants. This study reports the potential of callus from young leaf explants of *Clinacanthus nutans* forming somatic embryos and mature leaf explants producing friable callus for the establishment of cell suspension cultures in future bioactive compounds studies.

Key words: Callus induction, Auxin, Young leaves, Somatic embryos, Mature leaves, *Clinacanthus nutans*.

Abbreviations: 2,4-D = 2,4-Dichlorophenoxyacetic acid; FAA = Formalin- acetic acid-alcohol; MS = Murashige and Skoog medium; TBA = Tert-butyl alcohol

Introduction

Clinacanthus nutans (Burm. f.) *Lindau*, also known as Sabah Snake Grass, is a herbal plant belonging to the Acanthaceae family. *Clinacanthus nutans* is a small shrub found in deciduous forest of South East Asia. It is a well-known medicinal plant where its fresh leaves have long been used for various traditional treatments in Asia. The shape of the *Clinacanthus nutans* leaf is lanceolate with acute apex and pale green in color with short hair branches. Glandular trichrome and lithocyst components lie beneath the lower epidermis of the leaf. Its flowers are dull red in color with green and yellow streaks based on the lower lip located on the top branches with two stamens on the flower inserted in the throat. *Clinacanthus nutans* contains ovary that is compressed into two cells with each of them containing two ovules. Lumen of the xylem vessels in the group of xylem fibers are wide and arranged longitudinally while the phloem tissue located next to the xylem vessels lays in the parenchyma central portion in semi-circular. This plant can grow up to 1–3 meters in height (Kunsorn *et al*., 2013).

Clinacanthus nutans has been used traditionally in Thailand for viral treatment of herpes simplex (HSV) and herpes zoster where leaf extracts of this plants showed signs of suppression activity towards HSV-1 (Tuntiwachwuttikul *et al*., 2004; Vachirayonstien *et al.,* 2010). Other than that, the extracts of the *Clinacanthus nutans* leaves have also been utilized for traditional treatment of inflammation, rash treatment, scorpion and snake bites or as remedies for skin problems (Yoosook *et al.*, 1999; Pannangpetch *et al.,* 2007; Wanikiat *et al.,* 2008; Sakdarat *et al*., 2009; Vachirayonstien *et al.*, 2010; Kongkaew & Chaiyakunapruk, 2011; Yong *et al.,* 2013). *Clinacanthus nutans* in the form of herbal tea has also been used in Thailand and Indonesia to treat dysuria, dysentery, fever, diabetes mellitus and diarrhoea (Uawonggul *et al*., 2011). The Chinese traditional healers acknowledge the use of *Clinacanthus nutans* in the regulation of menstrual function, anemia, jaundice and also as a pain soother. In addition to the various reports of *Clinacanthus nutans* extracts having the ability to treat a variety of diseases, current testimonials from cancer patients and medical practitioners have reported the ability of its extracts to suppress the growth and advancement of cancer particularly in prolonging the life of cancer patients at critical stages. A recent report by Yong *et al.* (2013) reported on the antioxidant and antiproliferative characteristics of *Clinacanthus nutans* extracts acting against selected cancer cell lines. This study has proven the potential of *Clinacanthus nutans* to be used as an efficient alternative side treatment for cancer.

The bioactive chemical compounds associated to the medicinal properties of the *Clinacanthus nutans* extracts have been discovered to relate to chlorophyll a and chlorophyll b compounds. They are 132-hydroxy-(132S)-chlorophyll b, 132-hydroxy-(132R)-chlorophyll b, 132-hydroxy-(132S)-phaeophytin b, 132-hydroxy-(132R)-phaeophytin b, 132-hydroxy-(132S)-phaeophytin a, 132-hydroxy-(132R)-phaeophytin a, purpurin 18 phytyl ester and phaeophorbide where five of these identified compounds are novel compounds (Sakdarat *et al*., 2009). These compounds were found associated to the biochemical treatment of herpes virus through deactivation and suppression of viral activity during the early stages of infection (Vachirayonstien *et al.,* 2010).

These reports expanded the possibilities of further harnessing and studying other novel biochemical compounds in the extracts of *Clinacanthus nutans* that may exist either as single or in concert.

Currently, *Clinacanthus nutans* is not widely studied in plant tissue culture and there are no scientific reports on *In vitro* tissue and cell suspension culture for the purpose of harnessing its bioactive medicinal compounds. This study was conducted to investigate the optimal concentrations of 2,4-D for callus induction using leaf explants of *Clinacanthus nutans.* Histological analysis of callus was also performed to observe the characteristics of the callus induced.

Materials and Methods

Plant materials and media composition: *Clinacanthus nutans* explants were collected from Sungai Petani, Kedah, Malaysia. Two different sections of the plant, namely the young and mature leaves were used as explants for the establishment of *In vitro* culture of *Clinacanthus nutans*. The young leaves were taken from first and second node from the apex of the plant whereas mature leaves were obtained from third and fourth node.

The basal media used in this experiment is Murashige and Skoog medium (MS) (1962) supplemented with different concentrations of auxin, 2,4-Dichlorophenoxyacetic acid (2,4-D). High concentrations varied from 2.0, 4.0, 6.0, 8.0 and 10.00mg/L and low concentrations varied from 0.25, 0.50, 0.75 and 1.00mg/L were selected to be tested in this experiment.

Surface sterilization of explants: The leaf explants were surface sterilized using 2% dish washing liquid (Sunlight) and rinsed under running tap water for 5 minutes to remove all the surface detritus. Leaf explants were then transferred into 70% ethanol for 1 minute, rinsed with sterile distilled water and gently agitated with 20% (v/v) Clorox® for 10 minutes. Explants were then washed thoroughly with sterile distilled water and dried on sterile filter paper prior to culture.

Callus induction: The surface sterilized explants were cultured in MS media supplemented with 2,4-D at concentrations ranging from 0.25 mg/L up to 10.00 mg/L. The cultures were placed in the culture room and maintained at 25± 2ºC under cool white fluorescent light with the intensity of 3000 lux at 16 hours photoperiod. Fresh weight of callus were measured at week 15 and were analyzed using one way analysis of variance (ANOVA), followed by comparison of means using Tukey Test (HSD) at $p \leq 0.05$ (SPSS version 20).

Histological imaging and analysis: Callus clumps were excised from explants and immersed in FAA (formalin-acetic acid-alcohol) solution for one week. FAA solution was removed from callus by rinsing three times with distilled water. Callus were then transferred through a series of tert-butyl alcohol (TBA) (50%, 70%, 85%, 98%, and 100%) for one hour in each solution and further dehydrated in absolute TBA overnight before being transferred to xylene for 10 minutes and xylene/wax mixture for 30 minutes. Callus samples were infiltrated with molten Shandon Histoplast Pelletised Paraffin Wax (Thermoscientific, UK) for three times, 30 minutes each time at 60°C in an oven and were permanently casted in wax blocks using Leica EG1160 tissue embedding station (Nussloch, Germany). Callus blocks were sectioned 8 µm thick with a Leica RM2135 rotary microtome (Nussloch, Germany) and stained with toluidine blue. Slides were examined under Brightfield BX50 Olympus microscope (Olympus, Japan) and images were captured with JVC K-F55B camera (JVC Victor Company, Japan) and analysed using Soft Imaging System DocuVersion 3.1 image analysis system (Munster, Germany).

Results

The morphogenetic response of *In vitro* leaf explants of *Clinacanthus nutans* plants were significantly caused by various factors and conditions applied in this study. Callus induction of *Clinacanthus nutans* was performed using 2,4-D at various concentrations to evaluate the efficiency and ability of this auxin to induce the formation of callus using two different types of leaf explants. Callus was successfully initiated directly from excised surfaces of leaf explants placed in MS media supplemented with 2,4-D concentrations below 1 mg/L. However, it was evident that concentrations above 1 mg/L resulted in browning of explants with no proliferation of callus towards week 15. On the other hand, MS medium supplemented with 0.5 mg/L 2,4-D was found to induce the highest callus formation in young leaf explants with the mean of 6.230 g ± 0.909. As for the mature leaves of *Clinacanthus nutans*, MS media supplemented with 0.25mg/L 2,4-D was found to produce the highest callus fresh weight with an average of 3.575 g ± 0.628. Table 1 represents the percentage of callus induction and mean of callus fresh weight for all 2,4-D treatments on *Clinacanthus nutans* leaf explants. The morphology of the callus induced for all 2,4-D treatments were pale yellow in color with friable texture (Fig. 1). The mean of callus fresh weight induced from the leaf explants were found to be significantly different between both young and mature leaves (Fig. 2).

Table 1. Percentage of explants forming callus and average callus fresh weight of *Clinacanthus nutans* leaf explants in 2,4-D after 15 weeks of culture.

Concentration (mg/L)	Percentage of explants formation callus (%)		Average fresh weight (g) ± SE	
	Young leaves	Mature leaves	Young leaves	Mature leaves
0	0	0	0	0
0.25	100	90	1.761 ± 0.283	3.575 ± 0.628
0.50	100	90	6.230 ± 0.909	0.571 ± 0.058
0.75	100	100	0.998 ± 0.310	0.617 ± 0.051
1.00	40	100	0.185 ± 0.121	0.362 ± 0.181
2.00	40	40	/	/
4.00	0	20	/	/
6.00	0	0	/	/
8.00	0	0	/	/
10.00	0	0	/	/

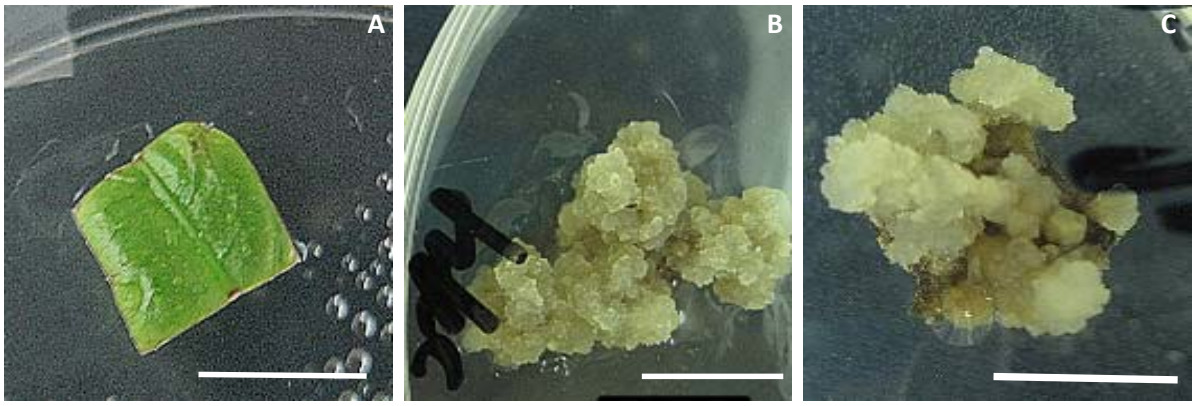

Fig. 1. The induction of callus from different leaf explant sources at 15 weeks of culture. *Scale bars* represent 1 cm.
A) Leaf explant in MS0. B) Formation of friable callus from young leaf explant in MS medium supplemented with 0.5 mg/L 2,4-D. C) Formation of friable callus from mature leaf explant in MS medium supplemented with 0.25 mg/L 2,4-D.

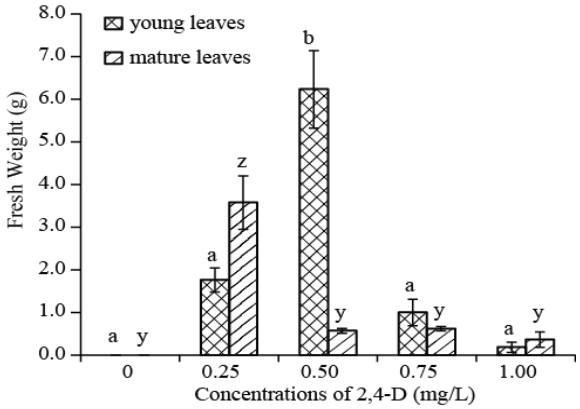

Fig. 2. Callus induction from leaf explants of *Clinacanthus nutans* for different concentrations of 2,4-D at 15 weeks of culture. Mean value of the callus fresh weight followed by same alphabet were not significantly different (Turkey Test, $p \leq 0.05$). *Error bars* represent mean ± standard error.

Histological analysis was performed on callus formed from the young leaf explants of *Clinacanthus nutans* treated with 0.5 mg/L 2,4-D and mature leaves at 0.25 mg/L 2,4-D. Callus formed was friable in structure and histological analysis revealed irregular cell size and shape throughout the callus mass (Fig. 3A). Morphologically friable section of the callus was composed of large cells with thin cell wall, forming a distinctive layer. This was easily distinguished from the cells at the centre of the callus mass that were more compact and smaller in size, indicating region of actively-dividing cells (Fig. 3B). Somatic embryos were found in the core of callus mass of young leaf (Fig. 4A). The external layer of the embryo consisted of two to three layers of meristematic cells. The inner cells of the embryo were large polyhedral in shape and parenchymatous in nature (Fig. 4B). The presence of mitotic cells zone in the callus of mature leaf and young leaf resembled clumps of meristemoids possibly developed from division of single cells (Figs. 3C, 4C). With reference to the histological evidence in this study, meristemoids present in the callus indicated potential formation of somatic embryos from young leaf explants of *Clinacanthus nutans*.

Discussion

The auxin 2,4-D is an effective plant cell regulator in inducing cell elongation and enlargement commonly used for callus induction. In the present study, all concentrations of 2,4-D at levels above 1 mg/L explants failed to induce viable callus with significant amount of fresh weight. The elevation of plant hormone concentrations was previously reported to have growth effects on the rate of callus formation in *Withania somnifera* (Chakraborty *et al.,* 2013). This result was also observed by Amiri *et al.* (2013) where the percentage of callus formation for *Solanum tuberosum L.* decreased as concentrations of 2,4-D increased above 3mg/L. The same results were observed in our investigation, where *Clinacanthus nutans* leaf explants displayed decreasing percentage of callus formation as the concentration of 2,4-D increased. This observation could be due to the nature of 2,4-D having the ability to function as a herbicide as reported by Barnes & Seefeldt (2009) where 2,4-D was found efficient in controlling the growth of woody and herbaceous broadleaf plants. Herbaceous plants susceptible to the auxin herbicides do not exhibit sufficient detoxification resulting in the prevention of normal nucleic acid metabolism and protein synthesis leading to plant death. Davis (1997) also reported 2,4-D having auxin-like properties at low concentrations and functioning as a herbicide at high concentrations. The herbicide-like properties have resulted in the browning and reduced viability of the *Clinacanthus nutans* leaf explants when placed in media with high levels of 2,4-D.

The observation from this study indicated that young leaf explants of *Clinacanthus nutans* cultured on MS medium supplemented with 0.5mg/L 2,4-D to be the most optimal in inducing higher amount of callus. This was similar to the work of Gopi and Vatsala (2006) that reported on leaf explants of *Gymnema sylvestre* cultured in MS medium supplemented with 0.5mg/L 2,4-D inducing maximum growth of callus. Ljung *et al.* (2001) discovered that the highest concentration of endogenous auxin IAA is located in vigorously proliferating young leaves and its levels decreases as the leaf expands (Ljung *et al*., 2001). This statement clearly indicates that the mechanism of auxin biosynthesis is regulated by tissue-specificity. Endogenous auxin in young organs of the plant require less exogenous auxin for cells that are actively dividing thus lower auxin levels are sufficient to induce callus formation.

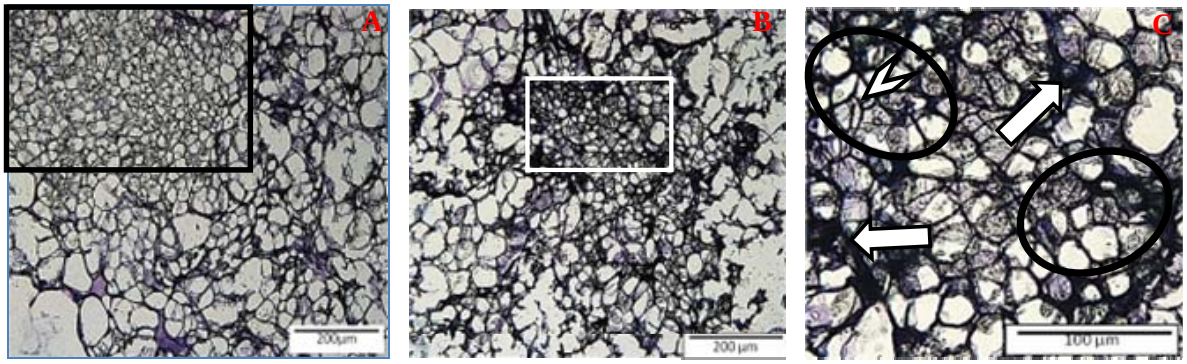

Fig. 3. Histology analysis of mature leaves callus at 15 weeks of culture.
A) Callus mass with two distinct regions of cells in different sizes. Differentiating inner region with mitotic cells zone (MCZ) is indicated in *window*. Beyond the mitotic cell zone (MCZ) are the non-differentiated cells which provided friable morphology of the callus. B) Inner region of callus with compact cells surrounded by large polyhedral parenchymatous cells. C) Enlargement of compact cells region (in *window*) reveals meristemoids zone (*circles*) possibly developed from division of single cells. *Arrow* indicates fibrillar and reticular filling within callus tissue. *Arrowhead* indicates visible nucleus within condensed cytoplasm.

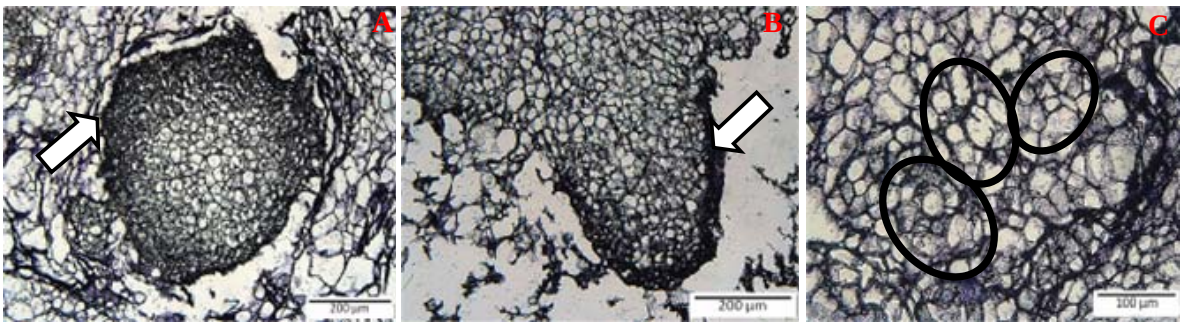

Fig. 4. Histology analysis of young leaves callus at 15 weeks of culture.
A) Somatic embryo in the core of callus mass, surrounded by undifferentiated cells. *Arrow* indicates small-size mitotic cells with dense cytoplasm at peripheral region of the somatic embryo, in contrast to large cells found in the centre of the somatic embryo. B) Nodular morphogenic callus with small mitotic cells in the peripheral region. C) Higher magnification: meristemoids zone (*circle*) preceding the formation of somatic embryos.

Histological analysis from this study indicated that callus produced from young leaves can potentially develop into somatic embryos. Several studies have also reported the possibilities of somatic embryos developing from single cells (Choudhary & Chin, 1995; Tokuhara & Mii, 2003; Jheng *et al*., 2006). Lee *et al.* (2013) provided image of light micrographs to clearly illustrate the development of protocorm-like bodies, a form of somatic embryo in orchids from isolated single cell of friable callus. Similar observations have been made by Palama *et al.* (2010) in protocorm callus of *Vanilla planifolia* and Lee *et al*. (2013) in protocorm-like bodies of *Phalaenopsis*. Different methods have been used to stimulate the development of somatic embryos. Zimmerman (1993) defined somatic embryogenesis as differentiation of somatic cells, under the right conditions into organized plants through embryogenic stages. Typical approach to obtaining somatic embryos involves transferring callus induced from auxin-rich medium under light exposure to basal medium without plant growth regulators (Ammirato, 1983; Chin *et al*., 1990). Alternatively auxins can be used to supplement basal MS medium in the induction of somatic embryos. Of all auxins used *In vitro*, 2,4-D is often found to be the most efficient plant growth regulator for the induction of somatic embryogenesis (Fehér *et al.,* 2003; Raghavan, 2004; Chen *et al.,* 2010). Rathore *et al.* (2014) induced somatic embryos from callus of *Salicornia brachiata* Roxb. in MS medium supplemented with 0.25 mg/L 2,4-D. Simoes *et al.* (2010) used ten-fold reduced 2,4-D concentration used for callus induction of *Cleome rosea* Vahl to develop somatic embryos. In some cases, auxin such as indole-3-butyric acid (IBA) has also been used for the induction of embryogenic callus in *Gossypium hirsutum* (Jingyan *et al.,* 2009). The use of 2,4-D in this study has not only successfully induced the formation of friable callus but also the potential development of somatic embryos from the leaf explants of *Clinacanthus nutans*.

Conclusion

The results from this study indicated that the callus induction of *Clinacanthus nutans* was found to be explant and 2,4-D concentration dependent. Young leaf explants of *Clinacanthus nutans* was discovered to produce higher

amounts of callus biomass and has the potential to develop into somatic embryos in comparison to mature leaf explants. MS media supplemented with 0.5 mg/L 2,4-D was found to induce high friable callus biomass in young leaves whereas MS media supplemented with 0.25 mg/L 2,4-D induced high friable callus biomass in mature leaf explants. The formation of somatic embryos alongside callus is the first to be reported in *Clinacanthus nutans*. These evidences will aid future *In vitro* studies on the establishment of cell suspension culture for consistent harvesting of secondary metabolites, bioactive metabolite analysis and also further investigation of the potential of this plant in the production of anti-cancer compounds found in this tropical medicinal plant.

Acknowledgments

The authors would like to acknowledge Universiti Sains Malaysia for funding the project under the Fundamental Research Grant Scheme (FRGS).

References

Amiri, M., M. Gerdakaneh, R. Mamghani and F. Nouri. 2013. Effect of different concentration of 2,4-D on callus induction and callus root indication in 2 explants from true potato (*Solanum tuberosum* L.) seeds. *Sci. J. Agron. & Plant Breed.,* 1(2): 56-63.

Ammirato, P.V. 1983. Embryogenesis. In: *Handbook of plant cell cultures, Volume I*. (Eds.): D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yamada. Macmillan, New York, pp. 82-123.

Barnes, D.L. and S. Seefeldt. 2009. Attenuation and effectiveness of triclopyr and 2,4-D along alaska highway right-of-ways in a continental and a coastal subarctic environment. *Dissertation, Alaska Department of T. Environment*, 2: 07-06.

Chakraborty, N., D. Banerjee, M. Ghosh, P. Pradhan, N.S. Gupta, K. Acharya and M. Banerjee. 2013. Influence of plant growth regulators on callus mediated regeneration and secondary metabolites synthesis in *Withania somnifera* (L.) Dunal. *Physiol. & Mol. Bio. Plants,* 19(1): 117-125.

Chen, A.H., J.L. Yang, Y.D. Niu, C.P. Yang, G.F. Liu, C.Y. Yu and C.H. Li. 2010. High-frequency somatic embryogenesis from germinated zygotic embryos of *Schisandra chinensis* and evaluation of the effects of medium strength, sucrose, GA3, and BA on somatic embryo development. *Plant Cell, Tiss. & Org. Cul.,* 102: 357-364.

Chin, Y.L., N. Greg and W. Terese. 1990. Efficient, direct plant regeneration from stem segments of chrysanthemum (*Chrysanthemum morifolium Ramat. cv. Royal Purple*). *Plant Cell Reports,* 8: 773-736.

Choudhary, M.L. and C.K. Chin. 1995. Somatic Embryogenesis in Cell culture of Carnation (*Dianthus caryophyllus.* L.). *Plant Growth Regulation,* 16(1): 1-4.

Davis, D.G. 1997. 2,4-Dichlorophenoxy acetic acid and indoleacetic acid partially counteract inhibition of organogenesis by difluoromethylornithine. *Physiologia Plantarum*, 101: 425-433.

Fehér, A., T.P. Pasternak and D. Dudits. 2003. Transition of somatic plant cells to an embryogenic state. *Plant Cell, Tiss. & Org. Cul,* 74: 201-228.

Gopi, C. and T.M. Vatsala. 2006. *In-vitro* studies on effect of plant growth regulators on callus and suspension culture biomass yield form *Gymnema sylvestre R.Br*. *Afr. J. Biotechnol.*, 5(12): 1215-1219.

Jheng, F.Y., Y.Y. Do, Y.W. Liauh, J.P. Chung and P.L. Huang. 2006. Enhancement of growth and regeneration efficiency from embryogenic callus cultures of *Oncidium* "Grower Ramsey" by adjusting carbohydrate sources. *Plant Sci.,* 170: 1133-1140.

Jingyan, S., L. Weiming, Z. Hanshuang, Z. Junli, Y. Xiaolin and W. Lian. 2009. Somatic embryogenesis and plant regeneration in glandless upland cotton (*Gossypium hirsutum* L.). *Frontiers of Agriculture in China*, 3(3): 279-283.

Kongkaew, C. and N. Chaiyakunapruk. 2011. Efficacy of *Clinacanthus nutans* extracts in patients with herpes infection: Systematic review and meta-analysis of randomised clinical trials. *Complementary Therapies in Medicine*, 19(1): 47-53.

Lee, Y.I., S.T. Hsu and E.C. Yeung. 2013. Orchid protocorm-like bodies are somatic embryos. *Am. J. Bot.,* 100(11): 2121-2131.

Ljung, K., R.P. Bhalerao and G. Sandberg. 2001. Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *The Plant J.,* 28(4): 465-474.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15: 473-497.

Palama, T.L., I. Fock, Y.H. Choi, R. Verpoorte and H. Kodja. 2010. Biological variation of *Vanilla planifolia* leaf metabolome. *Phytochemistry,* 71(5-6): 567-573.

Pannangpetch, P., P. Laupattarakasem, V. Kukongviriyapan, U. Kukongviriyapan, B. Kongyingyoes and C. Aromdee. 2007. Antioxidant activity and protective effect against oxidative hemolysis of *Clinacanthus nutans (*Burm.f) *Lindau*. *Songklanakarin J. Sci. & Technol.*, 29(1): 1-9.

Kunsorn, P., N. Ruangrungsi, V. Lipipun, A. Khanboon and K. Rungsihirunrat. 2013. The identities and anti-herpes simplex virus activity of *Clinacanthus nutans* and *Clinacanthus Siamensis*. *Asian Pasific J. Trop. Biomedicine.,* 3(4): 284-290.

Raghavan, V. 2004. Role of 2,4-dichlorophenoxyacetic acid (2,4-d) in somatic embryogenesis on cultured somatic embryos of arabidopsis: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. *Am. J. Bot.*, 91(11): 1743-1756.

Rathore, M.S., N. Paliwal, K.G.V. Anand and P.K. Agarwal. 2014. Somatic embryogenesis and *In vitro* plantlet regeneration in *Salicornia brachiata Roxb*. *Plant Cell, Tiss. & Org. Cul.,* 120: 355-360.

Sakdarat, S., A. Shuyprom, C. Pientong, T. Ekalaksananan and S. Thongchai. 2009. Bioactive constituents from the leaves of *Clinacanthus nutans Lindau*. *Bio-org. & Med. Chem.*, 17(5): 1857-1860.

Simoes, C., N. Albarello, C.H. Callado, T.C.D. Castro and E. Mansur. 2010. Somatic embryogenesis and plant regeneration from callus cultures of *Cleome rosea Vahl*. *Brazilian Arch. of Bio. & Technol.,* 53(3): 679-686.

Tokuhara, K. and M. Mii. 2003. Highly-efficient somatic embryogenesis from cell suspension cultures of *Phalaenopsis* orchids by adjustion carbohydrate sources. *In Vitro Cellular & Developmental Biology. Plant,* 39: 635-639.

Tuntiwachwuttikul, P., Y. Pootaeng-On, P. Phansa and W.C. Taylor. 2004. Cerebrosides and a mono-acylmonogalactosyl-glycerol from *Clinacanthus nutans*. *Chem. & Pharm. Bull.,* 52(1): 27-32.

Uawonggul, N., S. Thammasirirak, A. Chaveerach, C. Chuachan, J. Daduang and S. Daduang. 2011. Plant extract activities against the fibroblast cell lysis by honey bee venom. *J. Med. Plants Res.*, 5(10): 1978-1986.

Vachirayonstien, T., D. Promkhatkaew, M. Bunjob, A. Chueyprom, P. Chavalittumrong and P. Sawanpanyalert. 2010. Molecular evaluation of extracellular activity of medicinal herb *Clinacanthus nutans* against herpes simplex virus type-2. *Nat. Pro. Res.,* 24(3): 236-245.

Wanikiat, P., A. Panthong, P. Sujayanon, C. Yoosook, A.G. Rossi and V. Reutrakul. 2008. The anti-inflammatory effects and the inhibition of neutrophil responsiveness by *Barleria lupulina* and *Clinacanthus nutans* extracts. *J. Ethnopharmacol.,* 116(2): 234-244.

Yong, Y.K., J.J. Tan, S.S. The, S.H. Mah, G.C.L. Ee, H.S. Chiong and Z. Ahmad. 2013. *Clinacanthus nutans* extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines. *Evidence-Based Complementary and Alternative Medicine,* 2013: 1-8.

Yoosook, C., Y. Panpisutchai, S. Chaichana, T. Santisuk and V. Reutrakul. 1999. Evaluation of anti-HSV-2 activities of *Barleria lupulina* and *Clinacanthus nutans*. *J. Ethnopharmacol.,* 67(2): 179-187.

Zimmerman, J. L. 1993. Somatic embryogenesis: A model for early development in higher plants. *The Plant Cell*, 5: 1411-1423.

(Received for publication 24 March 2015)