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Callus Induction of *Ocimum sanctum* and Estimation of Its Total Flavonoids Content

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Abstract: *Ocimum sanctum*, also known as "Holy Basil" is one of the most common medicinal plants used by diverse cultures and tribal groups. Callus induction from leaf explants of *O. sanctum* was conducted by incubating leaf explants on Murashige and Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid (2,4-D), picloram, and indole-butyric acid (IBA) at 0, 1, 3, and 5 mg/L as well as the combination of 3 mg/L picloram with different concentrations (0, 0.5, 1.0, 1.5, and 2.0 mg/L) of 6-benzylaminopurine (BAP) or kinetin. Results obtained from the studies revealed that all the leaf explants incubated on phytohormone-supplemented medium formed callus. Leaf explants grown on 3 mg/L picloram formed callus after 8±1 days of culture, and degree of callus formation was found to be the highest (++++) among all the single auxin treatments. In contrast, the degree of callus formed from the leaf explants cultured on MS medium supplemented with combination of auxin and cytokinins were evidently lower than those in the single auxin treatments. Leaf explants cultured on kinetin-supplemented MS medium showed a higher degree of callus formation (++++) as compared to BAP-supplemented MS medium. There was no significant difference between the days of callus formation among all the cytokinin-supplemented treatments. The total flavonoids content of leaf-derived callus cultured on 3 mg/L picloram were also estimated and compared with the *in vivo* leaf tissues. The aluminium (III) chloride colorimetric assay revealed that the total flavonoids content of *in vivo* leaf tissues of *O. sanctum* were 2.2 times higher than leaf-derived callus, whereby the former produced 0.733±0.077 miligram catechin equivalent per gram fresh weight (mg CE/g fresh weight), while the latter only yielded 0.333±0.043 mg CE/g fresh weight.

Key words: Anti-oxidant, essential oil, Holy Basil, *Ocimum sanctum*, orientin, vicenin

INTRODUCTION

Ocimum sanctum, commonly known as "Holy Basil", belongs to the family of Lamiaceae. The plant is held sacred by Hindus all over the world as it is an herb that is used for religious purposes, in addition to its great medicinal values (Banu and Bari, 2007). Diverse medicinal properties of *O. sanctum* for example antidiabetic (Mukherjee *et al.*, 2006), antioxidant (Samson *et al.*, 2007), cardioprotection (Sood *et al.*, 2006), anti-fungal (Awuah and Ellis, 2002), immunostimulant (Mukherjee *et al.*, 2006) have attracted entrepreneurs to set eyes on this plant. The industry sector perceives *O. sanctum* not just as a spice used in household kitchen. They also see it as a potential lead to the production of drugs that are important to answer the insatiable needs of the population.

In order for *O. sanctum* to be used in the pharmaceutical industry to produce drugs that are beneficial, one outstanding obstacle, which is the standardization of quality and quantity of the compounds extracted from the plant itself has to be overcome. As discovered by various research, even close members of the same genus (*Ocimum*) do not possess the same chemical constituents (Singh and Sehgal, 1999). Razdan (2003) revealed that as *O. sanctum* is able to cross-pollinate with other plants of the similar genus, certain plants would not be true-to-type, and if there are genetic variation in the plant, the chemical constituents would be different. This is where plant tissue culture could be applied and helps to solve the problem, as plant tissue culture produces offspring that are identical to the parent plant. In consideration of the role that callus plays in micropropagation, as well as to estimate the potential of the usage of callus to extract secondary metabolites, hence the present study was carried out to identify the best treatment for callus induction from the leaf explants of *O. sanctum*. Apart from that, the total flavonoids content in leaf-derived callus and *in vivo* leaf tissues of *O. sanctum* was also compared.

MATERIALS AND METHODS

Plant Materials: Young leaves of *O. sanctum* were obtained from Sungai Buloh, Malaysia between October to December 2008.

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Surface Sterilization: Young leaves were placed in a clean beaker and were rinsed under running tap water for 30 minutes before the initiation of surface sterilization. The young leaves were immersed in 25% (v/v) Clorox® containing three drops of Tween-20 (Amresco, USA) for 10 minutes. The young leaves were then rinsed with sterile distilled water several times until all traces of Clorox® were eliminated. The sterilized young leaves were cut into 5 mm x 5 mm in size and were transferred to the medium with sterile forceps.

Basal Medium: Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as the basal medium. Sucrose at 3% (w/v) was added into the mixture. The pH of the medium was adjusted to 5.7±0.1 with 0.1 M HCl (Sigma Aldrich, USA) or 0.1 M NaOH (Sigma Aldrich, USA) followed by addition of 0.8% (w/v) agar. The medium was then autoclaved at 121ºC, 15 psi for 15 min. After autoclaving, a total of 25mL of the sterile medium was poured into 90 mm x 15 mm Petri dish in the laminar flow, and was allowed to solidify. The Petri dish was then sealed prior to the initiation of treatments.

Callus Induction in Single Auxin Treatments: In order to study the effects of various concentrations of different auxins on callus induction from the leaf explants, the MS basal medium was supplemented with different auxins. The auxins tested were 2, 4-Dichlorophenoxyacetic acid (2, 4 –D) (Sigma, USA), picloram (Duchefa, Netherlands) and Indole-butyric acid (IBA) (Duchefa, Netherlands), at the concentrations of 1, 3 and 5 mg/L. MS medium devoid of plant growth regulator was used as the control.

Callus Induction in Combination Treatments: The best auxin from the single auxin study, 3 mg/L of picloram was further combined with two cytokinins; 6-benzylaminopurine (BAP) (Duchefa, Netherlands) and kinetin (Duchefa, Netherlands) in order to study the effects of combination of auxin and cytokinin on callus induction. The concentrations of cytokinins examined were 0, 0.5, 1.0, 1.5 and 2.0 mg/L. The control for the experiment was MS medium lacked of plant growth regulator.

Culture Conditions: All the cultures were maintained in the culture room at 25±1ºC, under photoperiod of 16 hours light, 8 h dark provided by white fluorescent tubes with the intensity of 1000lux. The cultures were incubated for 30 days and daily observations were made to monitor the day of initial callus formation.

Data Collection: The day of initial callus formation, the morphology and colour of the callus were recorded. At the end the observation period, percentage of the explants forming callus as well as the degree of callus formation was measured.

Estimation of Total Flavonoids Content: In order to compare the total flavonoid contents between leaf-derived calli and *in vivo* leaves of *O. sanctum*, aluminium (III) chloride colorimetric assay was carried out. The samples for the biochemical tests were *in vivo* young leaves, and 3-month-old calli induced and maintained in MS medium supplemented with 3 mg/L of picloram.

A total of 0.5 g of samples were weighed and extracted with 50 mL of 80% (v/v) methanol (Merck, Germany). The mixtures were then ultrasonicated for 20 minutes followed by centrifugation at 12,000 revolutions per minute (rpm). Using a pipette, 1mL of the supernatant was collected into a test tube, and 4mL of deionized water was added. After that, 0.3 mL of 10% (w/v) $NaNO_2$ (Sigma, USA) were added to the test tubes, and was left to react for 5 minutes. Then, 0.3 mL of 10% (w/v) $AlCl_3$ (Sigma, USA) were added and was left for 1 minute to react. Lastly, 2 mL of 1M NaOH (Sigma, USA) was added and the mixtures were shaken. A total of 2 mL of the mixtures were transferred to a cuvette, and the absorbance values of both types of samples were measured using spectrophotometer (Thermo Electron Corporation, USA) at 510 nm. A mixture of 1mL of 80% (v/v) methanol, 4mL of deionized water, 0.3 mL of 10% (w/v) $NaNO_2$, 0.3 mL of 10% (w/v) $AlCl_3$ and 2 mL of 1M NaOH were prepared as the blank.

Catechin (Sigma, USA) was used as a standard in determining the total flavonoids content. From a catechin stock concentration of 100 mg/L, several dilutions were made to prepare a series of concentrations at 0, 10, 20, 40, 60, 80 and 100 mg/L. A standard curve was constructed with the optical density at 510 nm against the concentrations of catechin. The total flavonoids content of the samples were then estimated from the standard curve and further expressed in milligram of catechin equivalent per gram of sample fresh mass (mg CE/g FW).

Statistical Analysis: Each set of the experiment was repeated twice with three replicates and was subjected to statistical analysis. One way ANOVA and Tukey's Honestly Significant Difference (HSD) test ($p<0.05$) was used to determine the significant differences between means of the parameters that were recorded. Statistical analysis was performed using SPSS® software (Release 15.0) (SPSS Inc, USA).

RESULTS AND DISCUSSION

Callus Induction in Single Auxin Treatments: The manipulation of plant growth regulators is essential to optimize the induction of callus. After 4 weeks of observation, all the plant growth regulators tested on leaf explants showed 100% of callus formation (Table 1). However the degree of the callus induced from the leaf explants varied from each plant growth regulator. Apart from the differences between sizes of the callus formed, the day of callus formation varied noticeably. No somatic embryos or adventitious roots were formed during the duration of observation. Similarly, none of the leaf explants cultured on MS basal medium without plant growth regulator showed any sign of callus formation. In

Table 1: Callus induction from the leaf explants of *O. sanctum* after 4 weeks of culture in MS medium supplemented with different concentrations of auxins.

Auxins	Concentration (mg/L)	Day of Initial Callus Formation	Callus Formation (%)	Morphology and Colour of Callus	Degree of Callus Formation
Control	0	– [a]	0	–	–
2, 4-D	1	7 1 [b]	100	White, light greenish compact callus	+++
	3	8 1 [b]	100	White, light greenish compact callus	++++
	5	7 0 [b]	100	White, light greenish compact callus	++++
Picloram	1	8 1 [bc]	100	White, light greenish compact callus	++++
	3	8 1 [b]	100	White, light greenish compact callus	+++++
	5	7 1 [b]	100	White, light greenish compact callus	+++
IBA	1	13 4 [d]	100	Purplish, light greenish compact callus	+
	3	13 2 [cd]	100	Purplish, light greenish compact callus	+++
	5	9 2 [d]	100	Purplish, light greenish compact callus	++++

– = no callus formed, + = very few callus formation, ++ = minor callus formation, +++ = slight callus formation, ++++ = moderate callus formation, +++++ = profuse callus formation.Means within the column having the same letter were not significantly different by the Tukey HSD test ($p<0.05$).

Table 2: Callus induction from the leaf explants of *O. sanctum* after 4 weeks of culture in MS medium supplemented with 3 mg/L picloram and different concentrations of cytokinins.

Treatments	Day of Initial Callus Formation	Callus Formation (%)	Morphology and Colour of Callus	Degree of Callus Formation
Control	– [a]	0	–	–
3 mg/L picloram	8 1 [b]	100	White, light greenish compact callus	+++++
3 mg/L Picloram + 0.5 mg/L Kinetin	7 0 [b]	100	Light greenish compact callus	++++
3 mg/L Picloram + 1.0 mg/L Kinetin	7 0 [b]	100	Light greenish compact callus	++++
3 mg/L Picloram + 1.5 mg/L Kinetin	7 0 [b]	100	Light greenish compact callus	++++
3 mg/L Picloram + 2.0 mg/L Kinetin	7 0 [b]	100	Light greenish compact callus	++++
3 mg/L Picloram + 0.5 mg/L BAP	7 0 [b]	100	Light greenish compact callus	++
3 mg/L Picloram + 1.0 mg/L BAP	7 0 [b]	100	Light greenish compact callus	+++
3 mg/L Picloram + 1.5 mg/L BAP	7 0 [b]	100	Light greenish compact callus	++
3 mg/L Picloram + 2.0 mg/L BAP	7 0 [b]	100	Light greenish compact callus	++

– = no callus formed, + = very few callus formation, ++ = minor callus formation, +++ = slight callus formation, ++++ = moderate callus formation, +++++ = profuse callus formation. Means within the column having the same letter were not significantly different by the Tukey HSD test ($p<0.05$).

addition, the colour of all the leaf explants cultured on MS basal medium without auxin turned from green to brown in colour after three weeks of culture, and eventually died after 4 weeks of incubation.

Among the auxins tested, picloram exhibited 100% of callus formation on the leaf explants. All the calli from the picloram treatments were observed to be formed at the wounding site of the explants, the callus then continue to grow upwards until it finally covered the explant. The callus formed was compact, light green in colour with some white callus distributed on the top of the light green callus (Fig. 1). Picloram at 3 mg/L clearly outperformed its counterpart in the degree of callus formation. There was profuse callus formation (+++++) from the leaf explants cultured on 3 mg/L picloram-supplemented MS medium. As for explants that were incubated on other single auxin treatments, they did not form as much amount of callus as compared to 3 mg/L picloram. The leaf explants cultured on 3 mg/L picloram took 8 ± 1 days to form its first callus.

In the present study, an increasing trend could be observed in the degree of callus formation against the concentration of picloram supplemented. When the concentration of picloram was increased from 1 mg/L to 3 mg/L, the degree of callus formation was also increased. However, when the concentration of picloram was further increased to 5 mg/L, there was a decrement in the degree of callus formed. The decrement of the degree of callus formed could be due to the herbicidal property of picloram. In addition, high auxin concentrations promote the biosynthesis of ethylene by increasing the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (Kende, 1989). Ethylene is able to stimulate senescence of leaf, inhibit leaf abscission, and shoot growth.

The morphology of the callus formed in the treatments of IBA was clearly different from the callus formed in the treatments using other auxins. Leaf explants treated with picloram or 2, 4-D formed white light greenish coloured callus (Fig. 2). However, in IBA treatments, purplish along with light green coloured callus was produced (Fig. 3). The purple colour of the callus could be due to the accumulation of colour pigments, such as anthocyanin. Luczkiewcz and Cisowski (2001) reported that plant growth regulators (IAA and NAA) could stimulate the callus to synthesize anthocyanins and further increase the anthocyanin contents in the callus up to 44%.

Callus Induction in Combination Treatments: As preliminary studies showed that 3 mg/L of picloram induced the highest degree of callus from the leaf explant, therefore, it was used for further studies to evaluate the effects of combination of auxin and cytokinin on callus formation. After 4 weeks of observation, all the combination treatments on leaf explants showed 100% of callus formation (Table 2). In the treatment of 3 mg/L picloram combined with different concentrations kinetin, the leaf explants showed 100% of callus formation. The callus was formed from the cutting edges of the leaf explants and grown upwards to finally cover the explant. The callus established from the combination treatment of 3 mg/L picloram and various concentrations of kinetin were light green and compact in texture (Fig. 4). All the leaf explants formed callus after 7±0 days of culture. The combination of 3 mg/L picloram with kinetin at 0.5, 1.0, 1.5, and 2.0 mg/L formed almost similar sized callus. Moderate amount of calli (++++) were formed from the leaf explants.

In the combination of 3 mg/L of picloram with BAP, 100% of the leaf explants cultured formed callus after 7.0±0 days. The callus formed in BAP-supplemented medium was compact, and light green in colour (Fig. 5). After applying the combination treatment of auxin and

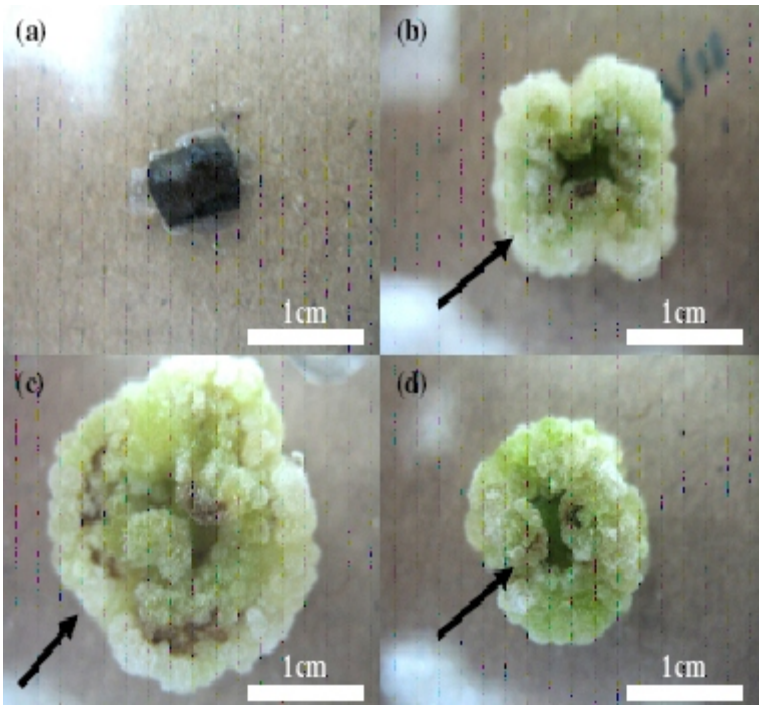

Fig. 1: Callus induction from the leaf explants of *O. sanctum* after 4 weeks of culture in MS medium supplemented with different concentrations of picloram. (a) 0 mg/L; (b) 1 mg/L; (c) 3 mg/L; (d) 5 mg/L. Arrow shows the callus formed on the explant.

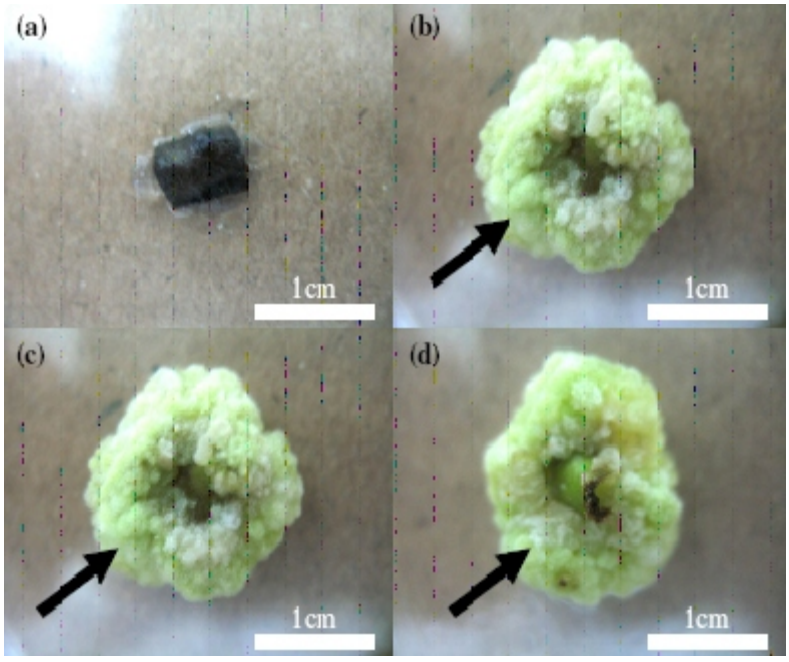

Fig 2: Callus induction from the leaf explants of *O. sanctum* after 4 weeks of culture in MS medium supplemented with different concentrations of 2, 4-D. (a) 0 mg/L; (b) 1 mg/L; (c) 3 mg/L; (d) 5 mg/L. Arrow shows the callus formed on the explant.

cytokinin, the degree of callus formation on the leaf explants were evidently lower than the degree of callus formation of the leaf explants incubated in the single auxin treatment. This could be due to the antagonist effect of exogenous cytokinin towards the production of indoleacetic acid oxidase isoenzyme in the callus cultures. Lee (1971) suggested that when exogenous IAA and cytokinins (kinetin and zeatin) were applied to tobacco callus cultures, the dry weight of the callus cultures significantly decrease with the increment of the

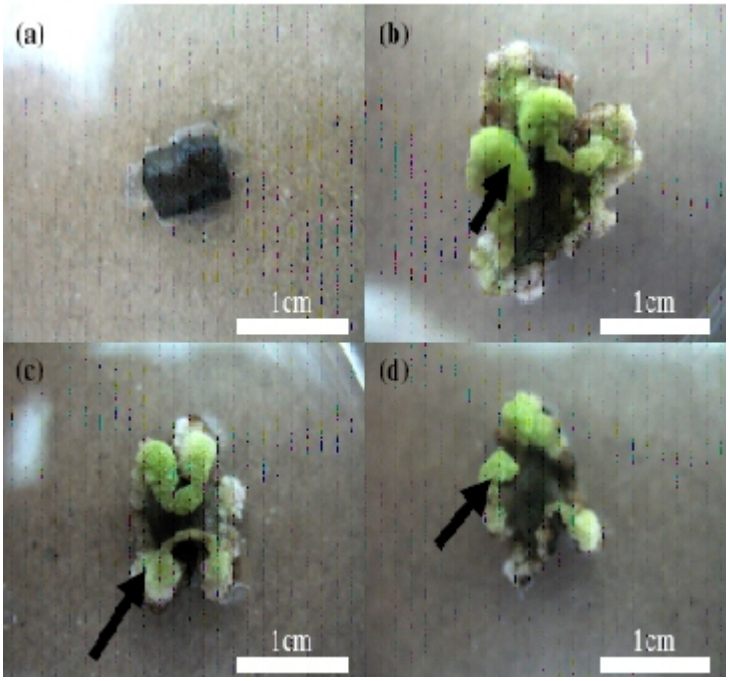

Fig. 3: Callus induction from the leaf explants of *O. sanctum* after 4 weeks of culture in MS medium supplemented with different concentrations of IBA. (a) 0 mg/L; (b) 1 mg/L; (c) 3 mg/L; (d) 5 mg/L. Arrow shows the callus formed on the explant.

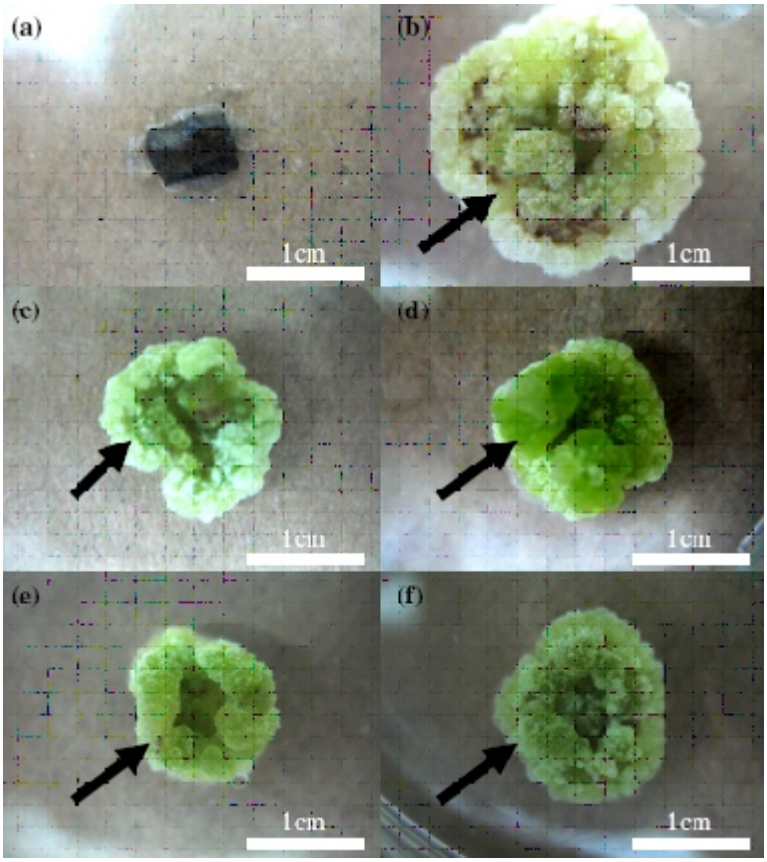

Fig. 4: Callus induction from the leaf explants of *O. sanctum* after 4 weeks of culture in MS medium supplemented with combination of 3 mg/L picloram and different concentrations of kinetin. (a) Control; (b) 0.0 mg/L kinetin (c) 0.5 mg/L kinetin; (d) 1.0 mg/L kinetin; (e) 1.5 mg/L kinetin; (f) 2.0 mg/L kinetin. Arrow shows the callus formed on the explant.

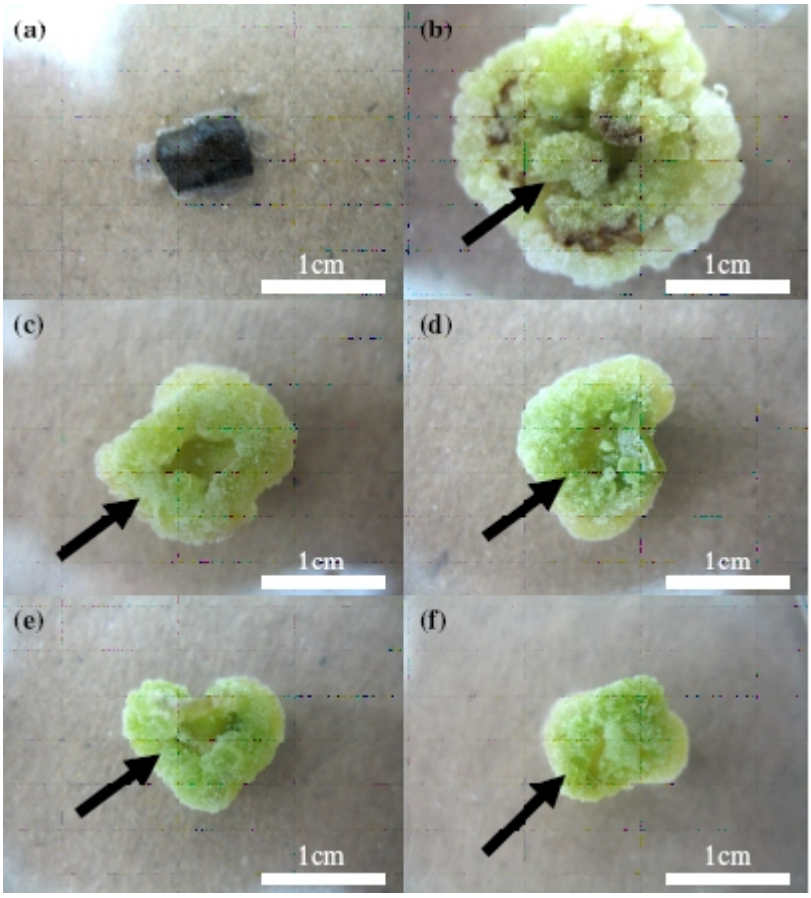

Fig. 5: Callus induction from the leaf explants of *O. sanctum* after 4 weeks of culture in MS medium supplemented with combination of 3 mg/L picloram and different concentrations of BAP. (a) Control; (b) 0.0 mg/L BAP (c) 0.5 mg/L BAP; (d) 1.0 mg/L BAP; (e) 1.5 mg/L BAP; (f) 2.0 mg/L BAP. Arrow shows the callus formed on the explant.

concentration of cytokinin applied. Lee (1971) further suggested that exogenous cytokinin is an inhibitor to the production of indoleacetic acid oxidase isoenzyme in the tobacco callus cultures. Low concentrations of cytokinin were believed to increase RNA and protein synthesis in plants. However, higher concentrations of cytokinin inhibited RNA and protein synthesis. Therefore, indoleacetic acid oxidase isoenzyme was inhibited, leading to the lacking of endogenous IAA produced by indoleacetic acid oxidase. Another possible reason that could contribute to this phenomenon of reduction in degree of callus formation from leaf explants when the explants were cultivated in combination treatment was due to a high concentration of exogenous auxins would stimulate the production of endogenous cytokinin (Einset, 1977). However, after taking into consideration of supplemented exogenous cytokinin, the overall ratio between auxin and cytokinin favoured the redifferentiation of callus cells into root cells (Chawla, 2002). During redifferentiation, the callus cells were no longer prepared for cell division, and rather ready to turn into root cells leading towards organogenesis (Mohr *et al*., 1995).

Table 3: Total flavonoids content (mg CE/g FW) in 3-month old leaf-derived callus and *in vivo* leaf tissue of *O. sanctum*

Sample	Total Flavonoids Content (mg CE /g FW)
Leaf-derived callus	0.333 0.043
In vivo leaf tissue	0.733 0.077

Estimation of Total Flavonoids Content: In this study, the total flavonoids content were successfully estimated using aluminium (III) chloride colorimetric assay whereby a total of 0.333 ± 0.043 mg CE/g FW of total flavonoids was determined in the leaf-derived callus of *O. sanctum* (Table 3). In the present study, the total flavonoids content of the *in vivo* leaf tissues were 2.2 times higher than the total flavonoids content of the leaf-derived callus. Calli are dedifferentiated cells, secondary metabolism in plant cells is tightly linked to its differentiation state. Thus, when a cell is completely de-differentiated, secondary metabolite pathways are often partially shut off. For instance, i*n vivo* leaf tissues of tobacco consist of differentiated cells and they are able to synthesis enzymes, like chalcone synthase, which contributes to the flavonoids biosynthesis pathway. However, callus generated from the leaf tissues, which contains partially dedifferentiated cells and dedifferentiated cells, is unable to produce as much enzymes like the *in vivo* leaf tissues.

Furthermore, the phase of cell growth in callus also influences the yield of flavonoid (Rahman, 1988). During early exponential phases of tobacco callus culture, anthraquinones were supposed to be the predominant secondary metabolites (Bohm and Stuessy, 2001). Similarly, during the early stationary phase, there will be a second maximum accumulation of secondary metabolites in cells (Bohm and Stuessy, 2001).

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REFERENCES

Awuah, R.T. and W.O. Ellis, 2002. Effects of some groundnut packaging methods and protection with *Ocimum* and *Syzygium* powders on kernel infection by fungi. Mycopathologia, 154(5): 264-269.

Banu, L.A. and M.A. Bari, 2007. Protocol establishment for multiplication and regeneration of *Ocimum sanctum* Linn.: an important medicinal plant with high religious value in Bangladesh. J. Plant Sci., 2(7): 530-537.

Bohm, B.A. and T.F. Stuessy, 2001. Flavonoids of Sunflower Family (Asteraceae). Springer, USA.

Chawla, H.S., 2002. Introduction to Plant Biotechnology. Science Publishers, USA.

Einset, J.W., 1977. Two effects of cytokinin on the auxin requirement of tobacco callus cultures. J. Plant Physiol., 59(3): 45-47.

Kende, H., 1989. Enzymes of ethylene biosynthesis. J. Plant Physiol., 91(1): 1- 4.

Lee, T.T., 1971. Cytokinin-controlled indoleacetic acid oxidase isoenzymes in tobacco callus cultures. J. Plant Physiol., 47(12): 181-185.

Luczkiewcz, M. and W. Cisowski, 2001. Optimisation of the second phase of a two phase growth system for anthocyanin accumlation in callus cultures of *Rudbeckia hirta*. Plant Cell, Tissue and Organ Culture, 65(11): 57-68.

Mohr, H., P. Schopfer, G. Lawlor and D.W. Lawlor, 1995. Plant Physiology. Springer-Verlag, Berlin, Heidelberg, pp: 198-230

Mukherjee, P.K., K. Maitib, K. Mukherjee and P.J. Houghton, 2006. Leads from Indian medicinal plants with hypoglycemic potentials. J. Ethnopharmacol., 106(28): 1-28.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15(3): 473-497.

Rahman, A., 1988. Studies in Natural Products Chemistry. Elsevier, Netherlands.

Razdan, R.Z., 2003. Introduction to Plant Tissue Culture. Science Publishers, USA.

Samson, J., R. Sheeladevi and R. Ravindran, 2007. Oxidative stress in brain and antioxidant activity of *Ocimum sanctum* in noise exposure. Neurotoxicology, 28(6): 679-685.

Singh, N.K. and C.B. Sehgal, 1999. Micropropagation of "Holy Basil" (*Ocimum sanctum* Linn.) from young inflorescences of mature plants. Plant Growth Regul., 29(5): 161-166.

Sood, S., D. Narang, M.K. Thomas, Y.K. Gupta and S.K. Maulik, 2006. Effects of *Ocimum sanctum* Linn. on cardiac changes in rats subjected to chronic restraint stress. J. Ethnopharmacol., 108(5): 423-427.