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# Induction and Microscopic Characterization of Globular Callus from Stem Explant of Labisia Pumila var. Alata

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**Abstract –** *Labisia pumila (BI.) F. Vill (Myrsinaceae) also known as Kacip Fatimah is a medicinal plant with an enormous therapeutics value and used widely for herbal industry in Malaysia. This study reports the induction of globular calli from stem explants of L. pumila var. alata. The embryogenic characteristics of the globular calli were further confirmed by morphological and histological analyses using the light microscope of sectioned material and scanning electron microscope. Globular callus was induced from stem explants that were pre-treated/un-pretreated with thidiazuron (TDZ) on Murashige and Skoog (MS) medium supplemented with 20 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and various amino acids (glutamine, arginine, proline and leucine) at the concentrations of 0.05, 0.1, 0.25 and 0.5 mM. After 6 weeks of incubation, explants pre-treated with TDZ induced 100% of globular callus while only friable calli were observed on un-pretreated explants. Addition of amino acids at concentrations between 0.05-0.25 mM enhanced induction of globular callus. Off all, 0.25 mM leucine provided the highest callus score (5.93±0.12) which produced 31 to 40 globular callus per explant. Double staining test shows the positive reaction to aceto-carmine red (0.5%) in the globular callus indicating the existence of embryogenic nature. Analysis under SEM and histological evaluation has confirmed the embryogenic characteristics with the appearance of isodiametric clump cells and containing prominent nuclei. The globular callus is further regenerated to shoots clump, thus, it is suggested that the use of TDZ-pretreatment method with addition of 20 µM 2,4-D and 0.25mM leucine successfully induced embryogenic callus from stem explant of L. pumila var. alata.* 

**Keywords**: Amino Acids; Labisis Pumila; Medicinal Plant; Somatic Embryo; Thidiazuron; Tissue Culture.

## 1.0 INTRODUCTION

*Labisia pumila* (Myrsinaceae) or commonly known as Kacip Fatimah is valued for its various medicinal properties, multiple therapeutic uses and potential pharmacological properties such as antioxidant, antibacterial and anticarcinogenic [1-3]. Exploitation of the species for commercial products is gradually expanding and led to a greater industrial request for the production of standardized plant material and extracts. Thus, an alternative method of propagation of the plant is crucial to ensure sustainable supply of the good standard quality raw materials. Plant tissue culture offer advantages of plant grown in control and standardized conditions, therefore, superior plant can be identified and clonally multiplied. Several studies have been reported on plant tissue culture of *L.pumila* such as *in vitro* seed

germination and establishment of *in vitro* plantlets of *L. pumila* var. *pumila* [4], comparative effects of plant growth regulators on leaf and stem explants of *L. pumila* var. *alata* [5] and adventitious root induction [6]. However, to date, no published information is available on the induction of somatic embryogenesis in *L. pumila*. Somatic embryogenesis is useful for rapid micropropagation and the production of mutants, artificial seeds and materials for the use of plant genetic engineering [7]. Thidiazuron (TDZ) or chemically known as N-phenyl-N'-l,2,3-thidiazol-5-ylurea is a substituted phenylurea compound which was developed for mechanized harvesting of cotton bolls and has now emerged as a highly efficacious bioregulant of morphogenesis in the tissue culture of many plant species. Application of TDZ induces a diverse array of cultural responses ranging from induction of callus to the formation of somatic embryos. TDZ exhibits the unique property of mimicking both auxin and cytokinin effects on growth. TDZ also induces different morphogenic responses of plant tissues by influencing the endogenous levels of both auxin and cytokinins [8]. TDZ, amino acid or their combination has been effectively used for the induction of somatic embryogenesis in species such as *Primulina tabacum* [9] and *Camellia nitidissima* [10]. Thus, this study was carried out to determine the effect of TDZ pre-treatment and amino acids on the globular callus induction from stem explants of *L. pumila* var. *alata*. The embryogenic properties of the globular callus were further characterized by microscopy analyses.

## 2.0 MATERIALS AND METHODS

### 2.1 Plant Materials and Explant Preparation

The plant materials obtained from Plant Tissue Culture Laboratory, Faculty of Science and Natural Resources, Universiti Malaysia Sabah. *Labisia pumila* var. *alata* plantlets were maintained on 0.8% (w/v) agar of Murashige and Skoog (MS) medium [11] supplemented with 3 % (w/v) sucrose, and sub-cultured monthly [4]. The stem nodes (~5 mm long) were excised from the 6-months-old plantlets and used for the subsequent experiments.

### 2.2 Explant Pre-Treatment with TDZ and Induction of Globular Calli from Stem Explant

Stem nodes were transferred into 250-mL Erlenmeyer flask containing 50-mL of 100 µM thidiazuron (TDZ) solutions for pre-treatment. The flasks were maintained on a rotary shaker (100 rpm) for 24 hours before placing the explants horizontally into the callus induction medium in petri dishes consist of 25-mL MS medium added with 3 % (w/v) sucrose, 0.8% (w/v) agar, 20 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and various concentrations (0.05,0.1,0.25 and 0.5 mM) of amino acids (arginine, proline, leusine and glutamine). The pH of the medium was adjusted to 5.6-5.7 prior to autoclaving at 121º C, 105 Kpa for 15 min. Amino acids were filter-sterilized and added into sterile media. Control explants (i.e. un-pretreated with TDZ or without amino acids) were also cultured in the same media. Each treatment conducted in five replicates with 5 explants per dishes, representing a total of 25 explants per treatment. Data such as percentage of callus induction, callus score and callus morphology were recorded at two weeks interval up to 6 weeks of incubation. All cultures were incubated in a plant growth chamber (SANYO, MLR-350H) at 25 ± 2º C with 16-h photoperiod provided by 36µmol $m^2$ $s^{-1}$ cool light fluorescent tube (SANYO, Japan).

### 2.3 Identification of Embryogenic Callus

After 6 weeks of culture, double staining technique was used to distinguish embryogenic cells from non-embryogenic cells as described by Silva et al. (2012) [12] with minor modification. Small pieces of the calli (50 mg) were collected and lightly macerated using a glass rod on top of a clean glass slide. After maceration, three drops of Evans-Blue (EB) dye (1%, w/v) was added. Excess of dye was removed and three drops of Aceto-Carmine (AC) dye (0.5%, w/v) were added and left to react for another three minutes. Excess of dye was removed and the cell mass was spread on a glass slide and analyzed under light microscope (Leica) at 10x and 40x magnification.

### 2.4 Scanning Electron Microscope Analysis

SEM observation was done according to Raha & Roy [13] with minor modification.The surfaces of the calli samples were cleaned with water and the area to be observed were selected and trimmed. Pieces of callus tissue were about the size <1cm in diameter. All samples were then air-dried to remove excess water. The dried samples were affixed into 30mm SEM mounting stubs made of aluminium with the help of conducive double adhesive copper tapes. The samples were then placed in an evacuation chamber of the SEM instrument (CARL ZEISS MA10) in vacuum at a pressure of $10^{-5}$ mm. The secondary electron image was observed in the CRT (cathode ray tube) and photomicrographs were taken at different magnifications.

### 2.5 Histological Analysis

Histological sectioning was performed according to Siang et al. [14] with minor modifications. Three samples of globular calli and non-globular calli were taken from selected replicates which contain of 0.05mM leucine for the histological study. Small clumps of calli were excised and fixed in fixative solutions, formalin-acetic acid-alcohol (FAA) in a ratio of 1:1:18, for 24 h. The tissues were then dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, 100%, v/v) for 20 min in each solution and embedded in paraffin wax. The material was sectioned 6 µm thick with a microtome (Shandon Finesse AS 325 Microtome) mounted on the slides and stained with 0.5% (w/v) of haematoxylin and eosin. The stained samples were observed under light microscope (Leica CME, Germany).

### 2.6 Experimental Design and Statistical Analysis

Experiments were conducted in a completely randomized design (CRD). The collected data were analysed by SPSS (Statistical Package for Social Science) version 11.5 and subjected to two way analysis of variance (ANOVA). The mean differences were tested using Duncan Multiple Range Test at $P< 0.05$.

## 3.0 RESULTS AND DISCUSSION

### 3.1 Effect of TDZ-Pretreatment and Amino Acids on Callus Induction

Three distinct types of callus morphology were observed in this study (Fig. 1), i.e, (i) type I – a combination of compact and globular callus induced on medium added with 2,4-D and pre-treated with TDZ; (ii) type II – Globular callus induced medium added with 2,4-D, amino acids and pre-treated with TDZ; and (iii) type III – friable callus induced from un-pretreated explants. Formation of callus on explant pre-treated with TDZ was observed after two weeks

of culture. The callus formed is white yellowish in color, translucent appearance and formation of structures similar to globular embryos (Fig. 2g-h and Fig. 3a). These structures indicate the first stage of embryogenic calli [15].

For explant pre-treated with TDZ, the presence of amino acids in the medium successfully promotes 100% induction of globular callus regardless of its concentration (Table 1). The mean score of callus enhanced with increasing concentration of amino acids from 0.05 mM up to 0.25 mM, and reduced at higher concentration (0.5 mM). Off all, explant treated with 0.25 mM leucine induced the highest callus score (up to 5.93 ± 0.12), which produced about 31 – 40 globular structures. Callus morphology mostly appears as compact and globular in the absence of amino acids. Meanwhile, explants without TDZ-pre-treatment failed to induce globular structures, although in the presence of amino acids. The induced callus is a yellowish and friable structure.

The results suggest the synergistic effect between TDZ, 2,-4-D and amino acid in the formation of globular structures and subsequently lead to somatic embryogenesis development in this species. Apart from mimicking both auxin and cytokinin effects on regulating cell development and growth, it is also believed that pre-treatment with TDZ at high concentration promote stress effects on the explant. Drastic changes in cellular environment such as exposing wounded cells or tissue to sub-optimal nutrient or hormone has been reported to generate significant stress effects and later promote cell differentiation towards the somatic embryo formation [16]. It was also inferred that application of TDZ enhance accumulation of minerals or other metabolites and predisposes the explant to stress [17]. 2, 4-D may also play a dual action in this culture, as this auxin is able to induce cells to increase endogenous IAA metabolism and as a stressor [18]. It is well known that endogenous IAA and ABA play a central role during the early phases of embryogenesis [16]. Cumulative roles by the plant growth hormones and their stress effects has mediated the signal transduction cascade and leads to the reprogramming of gene expression. This results in a series of cell divisions inducing either unorganized callus growth or polarized growth, which leads to somatic embryogenesis [19]. As for comparison, TDZ has been reported to promote an intermediate nodular and embryo-like callus structure in *Crocus sativus* L. [15] and could induce somatic embryogenesis in *Ochna integerrima* [20]. Amino acids such as arginine, glutamine and proline has been employed to promote embryogenesis as reported by Amali *et al*. (2014) [21] where proline, more stimulatory than arginine and glutamine in the formation of somatic embryo. In contrast, our result showed that lesion has a dominant effect on induction of globular callus. Amino acids increase the levels of reduced nitrogen that can stimulate somatic embryo development. The requirement for reduced nitrogen in embryo induction is that very young embryo lack nitrate reductase, which reduces nitrate to nitrite [22].

### 3.2 Identification of Embryogenic Callus by Double Staining with Aceto-Carmine (AC) and Evans Blue (EB)

The double staining analysis using AC and EB dyes confirmed the presence of the pro-embryogenic cells of the globular callus obtained in explants pre-treated with TDZ. High intensity of reactions area with AC dye in the globular callus indicate the densely cytoplasm characteristic which are more permeable to the red solution (Fig. 3b). Conversely, friable callus reacted less to AC dye and high intensity with Evans blue dye (Fig. 3f). According to Steiner *et al*. (2005) [23], the positive reaction to AC staining is associated with the competency of cells development and enhanced affinity to AC especially with the presence of small isodiametric cells, while EB affinity is mostly to elongated cells, and thus, double

staining with AC and EB is possible to discriminate the embryogenic cells. This result is in line with Pádua *et al.* (2014) [24], which observed an AC reactive in a small, and round (isodiametric) clusters (pro-embryogenic potential) of callus cultures in *Coffea arabica*, and Dias *et al.* (2014) [25] for the confirmation of the presence of pro-embryos in *Etlingera elatior* callus cultures.

**Table 1:** The effect of amino acids on globular callus induction from stem explant of *Labisia pumila* var. *alata* pre-treated with 100 µM TDZ on MS medium supplemented with 20 µM 2,4-D, 3 % (w/v) sucrose maintained under 16 h photoperiod at 25±2°C after 6 weeks of culture

| Amino acid | Concentration (mM) | Percentage of explant induce globular callus (%± SD) | Mean of score (±SD) | Time to induce callus (week) | Callus morphology |
|---|---|---|---|---|---|
| Control | | 100 ± 0.00$^{a}$ | 5.40 ± 0.53$^{abc}$ | 2 | Compact and globular |
| Arginine | 0.05 | 100 ± 0.00$^{a}$ | 4.80 ± 0.53$^{bcd}$ | 2 | Globular |
| | 0.1 | 100 ± 0.00$^{a}$ | 4.93 ± 0.46$^{bcd}$ | 2 | Globular |
| | 0.25 | 100 ± 0.00$^{a}$ | 5.20 ± 0.40$^{abc}$ | 2 | Globular |
| | 0.5 | 100 ± 0.00$^{a}$ | 4.40 ± 0.87$^{cd}$ | 2 | Globular |
| Proline | 0.05 | 100 ± 0.00$^{a}$ | 5.33 ± 0.50$^{ab}$ | 2 | Globular |
| | 0.1 | 100 ± 0.00$^{a}$ | 5.53 ± 0.31$^{ab}$ | 2 | Globular |
| | 0.25 | 100 ± 0.00$^{a}$ | 5.73 ± 0.23$^{ab}$ | 2 | Globular |
| | 0.5 | 100 ± 0.00$^{a}$ | 5.27 ± 0.31$^{abcd}$ | 2 | Globular |
| Leusine | 0.05 | 100 ± 0.00$^{a}$ | 5.53 ± 0.31$^{ab}$ | 2 | Globular |
| | 0.1 | 100 ± 0.00$^{a}$ | 5.73 ± 0.23$^{ab}$ | 2 | Globular |
| | 0.25 | 100 ± 0.00$^{a}$ | 5.93 ± 0.12$^{a}$ | 2 | Globular |
| | 0.5 | 100 ± 0.00$^{a}$ | 5.13 ± 0.83$^{abcd}$ | 2 | Globular |
| Glutamine | 0.05 | 100 ± 0.00$^{a}$ | 4.93 ± 0.42$^{bcd}$ | 2 | Globular |
| | 0.1 | 100 ± 0.00$^{a}$ | 5.00 ± 0.60$^{abcd}$ | 2 | Globular |
| | 0.25 | 100 ± 0.00$^{a}$ | 5.60 ± 0.20$^{ab}$ | 2 | Globular |
| | 0.5 | 100 ± 0.00$^{a}$ | 4.27 ± 0.42$^{d}$ | 2 | Globular |
| Type of amino acids (J) | | | F = 7.219 ** | | |
| Amino acid concentration (K) | | | F = 6.503 ** | | |
| (J) x (K) | | | F = 0.311 (NS) | | |

Note: Each value is mean of three replicates. Values followed by the same letter are not significantly different at P< 0.05 according to Duncan Multiple Range Test. SD-Standar deviation,**= Significance, NS= Not-significant.

Callus score : **0** = No changes, **1**= Swollen explants, **2**= 1-10 globular callus, **3**= 11-20 globular callus, **4**= 21-30 globular callus, **5**= 31-40 globular callus; **6**= 40 globular callus.

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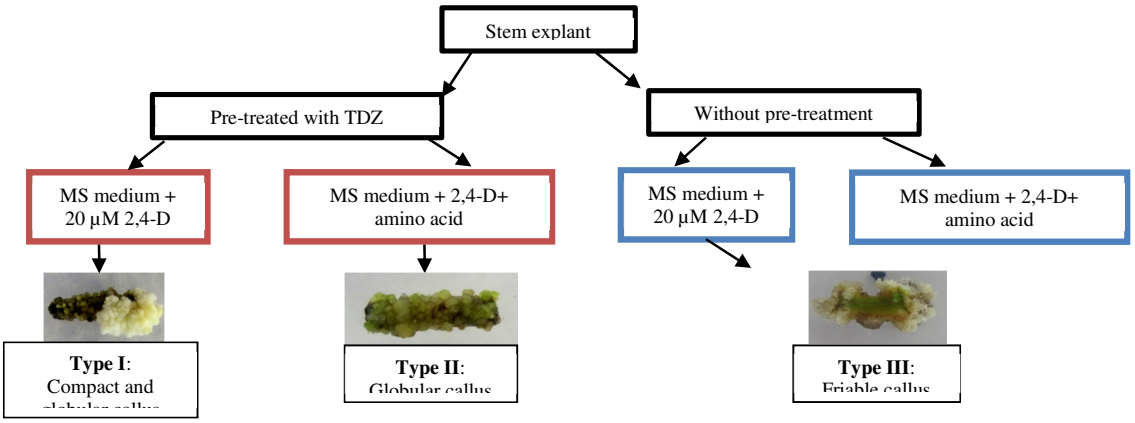


**Figure 1:** Simplified flow diagram of the morphologically distinct type of callus produced in this study

**Table 2**: The effect of amino acids on globular callus induction from stem explant of *Labisia pumila* var. *alata* without TDZ pre-treatment on MS medium supplemented with 20 µM 2,4-D, 3 % (w/v) sucrose maintained under 16 h photoperiod at 25±2ºC after 6 weeks of culture

| Amino acid | Concentration (mM) | Percentage of explant induce callus (%± SD) | Mean of score (±SD) | Time to induce callus (week) | Callus morphology |
|---|---|---|---|---|---|
| Control | | 100.00 ± 0.00$^{a}$ | 2.00 ± 0.2$^{ef}$ | 2 | friable |
| Arginine | 0.05 | 93.30 ± 11.60$^{ab}$ | 2.38 ± 0.38$^{cdef}$ | 2 | friable |
| | 0.1 | 100.00 ± 0.00$^{a}$ | 2.67 ± 0.12$^{abc}$ | 2 | friable |
| | 0.25 | 100.00 ± 0.00$^{a}$ | 2.93 ± 0.31$^{ab}$ | 2 | friable |
| | 0.5 | 86.67 ± 11.60$^{b}$ | 2.23 ± 0.25$^{cdef}$ | 2 | friable |
| Proline | 0.05 | 100.00± 0.00$^{a}$ | 2.20 ± 0.20$^{cdef}$ | 3 | friable |
| | 0.1 | 100.00± 0.00$^{a}$ | 2.33 ± 0.23$^{cdef}$ | 3 | friable |
| | 0.25 | 100.00± 0.00$^{a}$ | 2.40 ± 0.40$^{cdef}$ | 3 | friable |
| | 0.5 | 100.00 ± 0.00$^{a}$ | 1.93 ± 0.42$^{f}$ | 3 | friable |
| Leucine | 0.05 | 100.00 ± 0.00$^{a}$ | 2.27 ± 0.12$^{cdef}$ | 2 | friable |
| | 0.1 | 100.00 ± 0.00$^{a}$ | 2.33 ± 0.31$^{cdef}$ | 2 | friable |
| | 0.25 | 100.00 ± 0.00$^{a}$ | 2.47 ± 0.12$^{cde}$ | 2 | friable |
| | 0.5 | 100.00 ± 0.00$^{a}$ | 2.13 ± 0.12$^{def}$ | 2 | friable |
| Glutamine | 0.05 | 100.00 ± 0.00$^{a}$ | 2.53 ± 0.12$^{bcd}$ | 2 | friable |
| | 0.1 | 100.00 ± 0.00$^{a}$ | 2.93 ± 0.12$^{ab}$ | 2 | friable |
| | 0.25 | 100.00 ± 0.00$^{a}$ | 3.00 ± 0.20$^{a}$ | 2 | friable |
| | 0.5 | 93.30 ± 11.60$^{ab}$ | 2.35 ± 0.09$^{cdef}$ | 2 | friable |
| Type of amino acids (J) | | F = 2.83 (NS) | F = 10.5 ** | | |
| Amino acid concentration (K) | | F = (2.83 (NS) | F = 11.7** | | |
| (J) x (K) | | F = 1.26 (NS) | F = 0.5 (NS) | | |

Note: Each value is mean of three replicates. Values followed by the same letter are not significantly different at P< 0.05 according to Duncan Multiple Range Test. SD-Standar deviation,**= Significance, NS= Not-significant. Callus score : **0** = No changes, **1**= Swollen explants, **2**= callus grown on one side of explant , **3**= callus grown on two side of explant, **4**= callus

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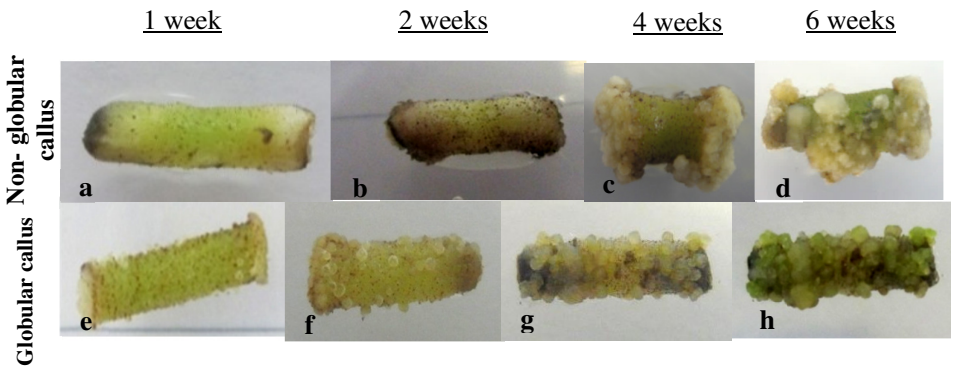


**Figure 2**: Induction of globular and non-globular callus from stem explant of *L.pumila* var. *alata*.

### 3.3 Scanning Electron Microscopy Study

SEM analysis was used to verify the morphological observation of the globular shape structures induced in stem explant of *L. pumila*. Under SEM analysis, it was observed that the cell masses developed under the influence of TDZ and amino acids had a rounded shape resembling the pro-embryo structure (Fig. 3c). The image presented in this figure indicates the individuality of globular structure (Gs). Similar results were also reported in callus culture of *Etlingera elatior* [25] and SEM analysis of embryogenic callus of sugarcane [26] that shows the formation of pro-embryos from a group of embryogenic cells in the embryogenic callus. The similarity of structure characteristics observed in this SEM analysis support the embryogenic potential of the globular callus induced in this study.

### 3.4 Histological Analysis and Somatic Embryo Development

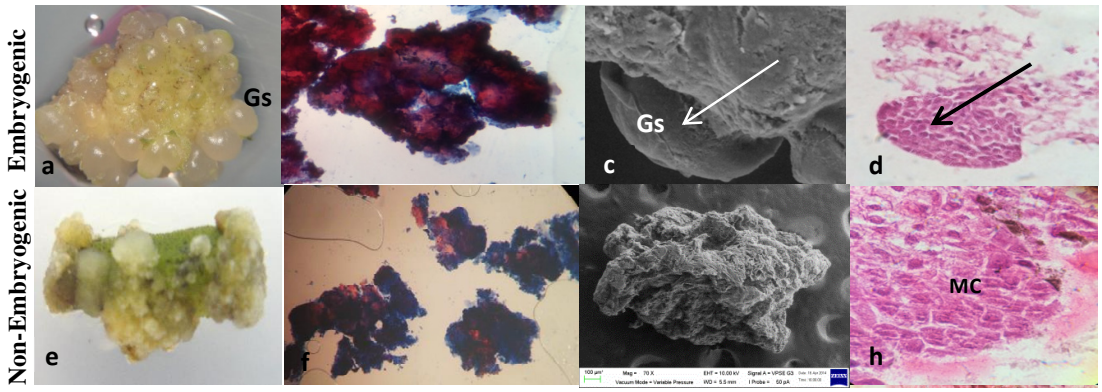


**Figure 3**: Histomorphological aspect of embryogenic and non-embryonic callus of *L. pumila* var. *alata* after 6 weeks of culture. (a) Globular structure produced on stem explant; (b) Double staining analysis with AC and EB showed deep red stained of globular structure cell; (c) SEM analysis of globular structure (Gs); (d) Histological examination showing globular somatic embryo with isodiametric clump of cells (arrow) containing prominent nuclei (40X magnification); (e) friable callus; (f) higher intensity of blue-stained on friable callus cell; (g) SEM analysis of friable callus; (h) histological analysis showing a meristematic center (MC) in a non-embryogenic callus.

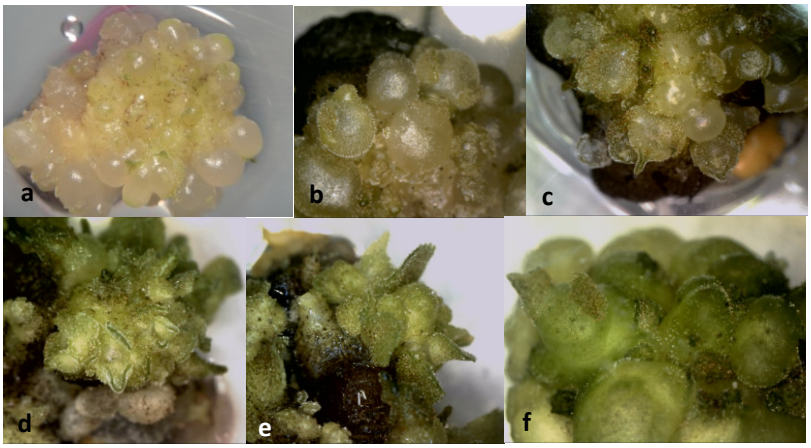


**Figure 4**: Shoot organogenesis and regeneration from globular embryogenic callus. (a – c) stem explant with globular structure distributed on the surface after 6 weeks in culture medium; (d) Globular embryogenic structures gradually turn green and form green shoot buds on the surface e-f) development of shoot buds from the globular embryogenic callus.

Histological analysis of the globular callus (Gs) section after six weeks of culture has detected the visible isodiametric cell with prominent nucleus and dense cytoplasm (Fig. 3d) which are the characteristic of embryogenic cells. Thus, we strongly suggest the embryogenic potential of this type of callus. The globular structures independent vascular tissue were also identified in this study which is in line with those reported in somatic embryogenesis of mangosteen [27]. The friable, non-embryogenic callus exhibited meristematic characteristics which designated as meristematic centers, MC (Fig. 3h). However, these cells were dispersed and did not form an organized structure like those observed in the embryonic cells (Fig. 3d), and therefore did not further develop to embryo. Regeneration of embryogenic callus to a new shoot buds were observed after leaving in the same induction medium for 2-3 weeks (Fig. 4). Yang et al. [9] also reported that the induced somatic embryos of *Primulina tabacum* could develop into shoot buds when left on the same induction medium.

## 4.0 CONCLUSION

In this study, we report successful induction of globular callus from stem explant of *L. pumila* var. *alata* pre-treated with TDZ and cultured on MS medium supplemented with 2,4-D and amino acids. Pre-treatment with TDZ was essential for promoting globular structures which subsequently identified as embryogenic callus. Microscopic observation, double staining test and histological analysis have confirmed the embryogenic characteristics of these globular structures, and thus we suggest for potential somatic embryogenesis in this species. We also suggest the synergistic effect between TDZ, 2,4-D and amino acid contributed to somatic embryogenesis event in this species. However, further research on developmental stages of somatic embryogenesis is necessary.

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