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## Induction of Somatic Embryogenic Callus From the Leaves of *Pereskia grandifolia*

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**Abstract:** A protocol was developed for the induction of somatic embryogenic calluses from the leaf explants of *Pereskia grandifolia*. The somatic embryogenic callus was best induced from the mid vein section of the leaf as compared to the leaf blade when they were cultured on B5 medium supplemented with  $6.0 \text{ mg L}^{-1}$  2,4-Dichlorophenoxyacetic acid (2,4-D). The mid vein section of the leaf taken from the middle of the leaf to the basal end near the petiole (MP) was found to form embryogenic calluses better than the mid vein explant taken from the middle part of the leaf towards the leaf apex (MA). MP explants produced  $0.652 \pm 0.078 \text{ g}$  of somatic embryogenic calluses when they were cultured on Gamborg medium (B5) supplemented with  $6.5 \text{ mg L}^{-1}$  2,4-D after 8 weeks of culture. The amount of embryogenic callus formed was not significantly different from that induced from the MP explants that were cultured on B5 medium supplemented with 6 or 7  $\text{mg L}^{-1}$  2,4-D. Histological study confirmed the formation of the embryogenic calluses from *P. grandifolia* leaf explants.

**Key words:** Gamborg medium (B5), *Pereskia grandifolia*, somatic embryogenic callus, leaf mid vein, 2,4-D

### INTRODUCTION

*Pereskia grandifolia* is a type of cactus plant belonging to the Cactaceae family and Pereskioideae subfamily. It has a woody stem with large fascicles of spines and leaves that are different from the other cacti species. *P. grandifolia* also known as 'Rose of Burma' and has been used by the Malaysian Chinese as traditional remedy for the prevention and treatment of uterus cancer and fibroids. The leaves extract was found to inhibit the cell growth of NCI-II23 (human non-small lung adenocarcinoma) and Caov-3 (human ovarian carcinoma) cell lines (Ooi *et al.*, 2003).

The conventional propagation method of *Pereskia grandifolia* is beset with problems of scanty and delayed rooting of seedlings and vegetative cuttings. This approach can hardly fulfill the increasing demand of the local market. *In vitro* culture technique could be the alternative method for the production of plant material as future commercial supplies of *P. grandifolia* for the preparation of herbal products. The production of somatic embryogenic calluses could be used as the material source and was fundamentally more efficient than root and shoots induction. The process is usually less elaborate and less diversity of media are required (Endress, 1994). Until today, the induction of somatic embryogenic callus has not been reported for any of

the *Pereskia* sp. This study hence reports the induction of embryogenic calluses from the different parts of the *P. grandifolia* leaves.

### MATERIALS AND METHODS

**Establishment of aseptic plant materials:** *P. grandifolia* plants were collected from the herbal garden of School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The young leaves were used as explants for the induction of embryogenic callus. The surface of the leaves was wiped with 35% ethanol followed by washing with 50% Clorox<sup>®</sup> (v/v) solution with the addition six drops of Teepol. They were then rinsed with running tap water for 30 min. The explants were then surface-sterilized with  $150 \text{ mg L}^{-1}$  Sodium dichloroisocyanurate (NaDCC) for 15 min. They were then rinsed three times with sterile distilled water. The mid-vein section of the leaf ( $1 \times 1 \text{ cm}$ ) and the leaf blade ( $1 \times 1 \text{ cm}$ ) were cut from the aseptic explants before inoculated onto solid Murashige and Skoog (1962) (MS) medium without any plant growth regulator. The pH of the medium was adjusted to 5.7-5.8 followed by the addition of  $7.5 \text{ g L}^{-1}$  agar (Chile Algas) before the medium was autoclaved using Elite Sterilizer EAC-4000C at  $1.05 \text{ kg cm}^{-2}$  and  $121^\circ\text{C}$  for 13 min. The cultures were placed in the culture room maintained at  $25 \pm 2^\circ\text{C}$  under continuous cool white fluorescent lights

with a photon flux density of 150-200 lux. The aseptic leave explants were then used for the induction of embryogenic callus.

**Induction of embryogenic callus:** The aseptic mid-vein section of leaf (1×1 cm) and the leaf blade (1×1 cm) were cultured on Gamborg B5 medium (Gamborg *et al.*, 1968) supplemented with 0, 2, 4, 6, 8 and 10 mg L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D). Six experimental units were used for each medium and each type of leaf explant. The experiment was carried out using the complete randomized block design. Each experimental unit consisted of three explants. Biomass of callus induced from each type of explant in each medium was determined after 8 weeks of culture. The data were analyzed using two-way analysis of variance (ANOVA) and the best callus induction medium was selected after comparison of mean using Tukey test at p = 0.05.

**Effect of different part of leaf Mid-vein section on induction of embryogenic callus:** The leaf mid-vein was cut into two sections: From middle of the leaf to the basal end near the petiole (MP) and from the middle part of the leaf towards the leaf apex (MA). These explants were cultured on B5 medium supplemented with 6 mg L<sup>-1</sup> 2,4-D, the optimum callus induction medium determined in the previous section. Twenty explants were used for each type of explant. The biomass of callus induced from the two different sections of leaf mid-vein was recorded after 8 weeks of culture. The data was analyzed using student t-test at p = 0.05.

**Optimization of culture medium for the induction of embryogenic callus:** The MP leaf explants were cultured on B5 medium supplemented with 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 mg L<sup>-1</sup> 2,4-D. Six experimental units with three explants per unit were used for each medium using complete randomized block design. Biomass of callus that was induced from each of the different medium was recorded after 8 weeks of culture. The data was analyzed using two-way Analysis of Variance (ANOVA) and the best medium was selected after comparison of mean values by Tukey test at p = 0.05.

**Histological study of embryogenic callus:** To study the developmental stages of the somatic embryogenic callus, the callus tissues were fixed in formaldehyde, glacial acetic acid and absolute alcohol in the ratio of 1:1:18 for 48 h. The fixed tissues were dehydrated in a series of tertiary butyl alcohol (TBA) (50, 70, 85, 98, 100% (v/v), absolute TBA) followed by submerging in xylene for 1-2 h. The samples were embedded in Paraplast Tissue

Embedding wax overnight. The wax blocks were sliced into 10 µm thickness sections which were then double stained with Safranin and Fast Green. The prepared slides were observed using the Olympus SZ-PT Phaco light microscope.

## RESULTS AND DISCUSSION

Healthy and aseptic leaf explants were used for the induction of embryogenic callus. The amount of calluses initiated from solid B5 medium supplemented with different concentration of 2,4-D (0, 2, 4, 6, 8 and 10 mg L<sup>-1</sup>) was significantly different when using the mid-vein section leaf explants but not for the leaf blade explants. More calluses were induced from the mid-vein section of the leaf cultured on B5 medium supplemented with 6 mg L<sup>-1</sup> 2,4-D (0.478 g) and B5 medium supplemented with 8 mg L<sup>-1</sup> 2,4-D (0.461 g). B5 medium supplemented with less than 6 mg L<sup>-1</sup> or more than 8 mg L<sup>-1</sup> 2,4-D induced less than 0.25 g of embryogenic callus from the mid vein section of *P. grandifolia* leaves after 8 weeks of culture. Only 0.1-0.22 g of callus was induced from the leaf blade explants that do not contain any leaf veins when they were cultured on B5 medium supplemented

Table 1: Production of embryogenic callus from the leaf explants of *P. grandifolia* on solid B5 medium supplemented with different concentration of 2,4-D after 8 weeks of culture

B5+2,4-D (mg L <sup>-1</sup> )	Callus biomass induced from Mid-vein of the leaf explants (g)±SE	Callus biomass induced from the leaf blade explants (g)±SE
0	0c	0y
2	0.125±0.073b	0.179±0.053x
4	0.245±0.108b	0.221±0.124x
6	0.478±0.068a	0.126±0.051x
8	0.461±0.181a	0.097±0.046x
10	0.179±0.045b	0.130±0.044x

Mean values within columns followed by same alphabet were not significantly different based on Tukey test at p = 0.05

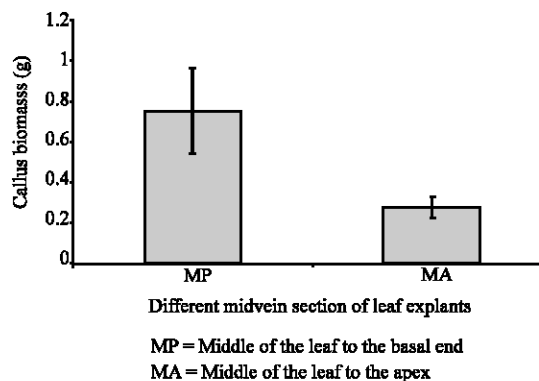


Fig. 1: Effect of different part of leaf mid-vein section on the induction of Embryogenic callus of *P. grandifolia*

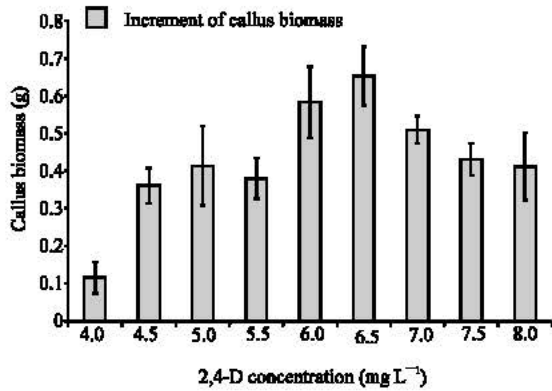


Fig. 2: Effect of 2,4-D supplemented into B5 medium on Induction of Embryogenic callus from the mid-vein of *Pereskia grandifolia* leaves

with 2-10 mg L<sup>-1</sup> of 2,4-D. Callus was not induced either from the mid-vein section of the leaves or the leaf blade of *P. grandifolia* when they were cultured on B5 medium without 2,4-D (Table 1).

The amount of calluses produced from different section of the leaf mid-vein was found to be significantly different. The MP section leaf explants produced the most callus (0.752±0.212 g) compared to the MA section (0.278±0.053 g) on B5 medium supplemented with 6 mg L<sup>-1</sup> 2,4-D (Fig. 1). Chen *et al.* (1999) had used different part of *in vitro* leaf explants of *Oncidium* Gower Ramsey to induce direct somatic embryos. Their research showed that the formation of the foliar embryos was affected by the maturity state of the leaves and more foliar embryos were formed on the older leaves. In induction of somatic embryogenic callus of *P. grandifolia*, various part of leaf did play a role in the formation of embryogenic callus

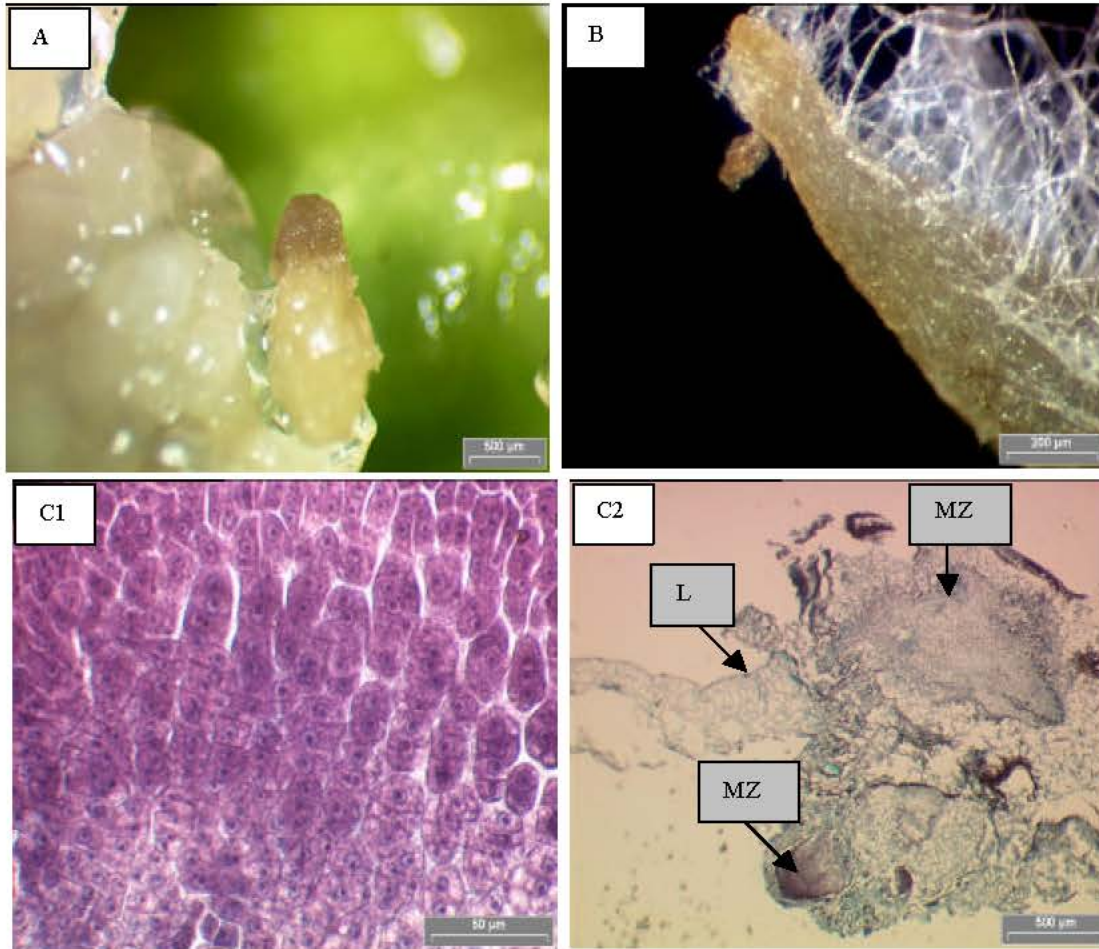


Fig. 3A-C: Embryogenic callus formed from the leaf explants of *P. grandifolia* when cultured on B5 medium supplemented with 2,4-D. (A) Nodular callus from leaf mid-vein; (B) Fine roots produced from embryogenic callus after 8 weeks of culture; (C) Histological section of embryogenic callus induced from leaf explants (L) showing active cell division (C1) and meristematic zone (MZ) (C2)

beside the effect of plant growth regulator, 2,4-D. This was because the mid-vein section of leaf explants consisted of more vascular tissue as compared to the tissue at the leaf blade.

In the optimization of medium study using the MP leave as explants, B5 medium supplemented with 6.5 mg L<sup>-1</sup> 2,4-D was found to induce a maximum biomass (0.652±0.078 g) of embryogenic callus within 8 weeks of culture and this was found to be not significantly different to the amount of embryogenic callus induced on B5 medium supplemented with 6 or 7 mg L<sup>-1</sup> 2,4-D (Fig. 2). Bhojwani and Razdan (1996) reported that 2,4-D (0.5-1 mg L<sup>-1</sup>) was generally used for the induction of embryogenic callus. However, Yemets *et al.* (2003) reported that 8 mg L<sup>-1</sup> 2,4-D was needed to be added into the MS basic medium together with 1 µM copper sulphate for the high-frequency production of somatic embryos of cassava for cryopreservation purpose. Chengalrayan and Gallo-Meagher (2001) also found that 13.6 µM 2,4-D was supplemented into MS basic medium to induce embryogenic calluses from the sugarcane young leaves (*Saccharum* sp. Hybriv cv. CP 84-1198). The amount of 2,4-D that was needed for induction of embryogenic calluses was totally depended on the sensitivity of the plant cell. As suggested by Arnold *et al.* (2002), the competence of embryogenic callus induction of each plant species was due to the sensitivity of the explant cells towards the various type of auxin. Kim *et al.* (2004) observed that embryogenic callus could be obtained from the hypocotyl segments of *Medicago sativa* L. using MS medium supplemented with 0.02 mg IAA and 1.0 mg L<sup>-1</sup> zeatin after 45 days of culture. While Kim *et al.* (2003) found that embryogenic callus could be propagated from the leaf explants of *Rosa hybrida* L. on MS medium with the presence of NAA, Zeatin and GA<sub>3</sub>. However, our experimental result indicated that for *P. grandifolia*, 2 mg L<sup>-1</sup> 2,4-D incorporated into the MS medium was sufficient to induce embryogenic callus from the leaf explants but 6.5 mg L<sup>-1</sup> of 2,4-D was needed to induce higher biomass of embryogenic calluses.

Histological study showed that no callus or nodular embryos were formed after one week of culture from the leaf blade explants that were cultured on B5 medium supplemented with 6.5 mg L<sup>-1</sup> 2,4-D but the tissues were swollen at the cutting edges. However, the embryogenic calluses were formed from the leaf mid-vein explants. The embryogenic calluses were light green in color and nodular in shape (Fig. 3A). Small fine roots were formed from the embryogenic callus surface after 5 weeks of culture in all B5 medium supplemented with 2,4-D (Fig. 3B). These nodular calluses were composed of non-vacuolated cells with central nuclei undergoing a rapid mitotic division (Fig. 3C1) and forming

meristematic cells zone (Fig. 3C2). These results supported our findings that mid-vein sections (MP) of *P. grandifolia* leaves were easier to form embryogenic calluses when they were cultured on B5 medium supplemented with 6.5 mg L<sup>-1</sup> 2,4-D compared to the leaf blade explants.

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#### REFERENCES

- Arnold, S.V., I. Sabala, P. Bozhkov, J. Dyachok and L. Filonova, 2002. Developmental pathways of somatic embryogenesis. *Plant Cell, Tiss. Organ. Cult.*, 69: 233-249.
- Bhojwani, S.S. and M.K. Razdan, 1996. *Plant Tissue Culture: Theory and Practice A Revised Edition*. Elsevier Science, New York.
- Chen, J.T., C. Chang and W.C. Chang, 1999. Direct somatic embryogenesis on the leaf explants of *cnidium* gower ramsey and subsequent Plant Regeneration. *Plant Cell Reports*, 19: 143-149.
- Chengalrayan, K. and M. Gallo-Meagher, 2001. Effect of Various Growth Regulator on Shoot Regeneration of Sugarcane. *In vitro Cell Dev. Biol. Plant*, 37: 382-387.
- Endress, R.H., 1994. *Plant Cell Biotechnology*. Springer-Verlag, Berlin, Heidelberg, Germany.
- Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.*, 50: 151-158.
- Kim, C.K., J.D. Chung, S.O. Jee and J.Y. Oh, 2003. Somatic embryogenesis from *in vitro* grown leaf explants of *Rosa hybrida* L. *J. Plant Biotechnol.*, 5: 169-172.
- Kim, Y.S., M.Y. Kim and M.S. Yang, 2004. Effect of plant growth regulators on plant regeneration through somatic embryogenesis of *Medicago sativa* L. *J. Plant Biotechnol.*, 6: 87-90.
- Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.
- Ooi, K.L., T.S. Tengku Muhammad and S.F. Sulaiman, 2003. Cytotoxic Effects of *Pereskia grandifolia* Extracts Against Different Classes of Cell Lines. *Proceedings of The 14th National Biotechnology Seminar, USM Penang, Malaysia*.
- Yemets, A.L., L.A. Klimkina, L.V. Tarassenko and Y.B. Blume, 2003. Efficient callus formation and plant regeneration of goosegrass [*Eleusine indica* (L.) Gaertn.]. *Plant Cell Report*, 21: 503-510.