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Effects of Plant Growth Regulators on the Biomass of Embryogenic Cells of *Cyperus aromaticus* (Ridley) Mattf and Kukenth

Zainah Daud and Chan Lai Keng
Plant Tissue and Cell Culture Laboratory, School of Biological Sciences,
Universiti Sains Malaysia, 11800 Penang, Malaysia

Abstract: High production of cell biomass and somatic embryos could be established in the suspension cultures of *Cyperus aromaticus*. The callus derived from root pieces was used for preparation of the cell suspension cultures. The cells pretreated with 2,4-Dichlorophenoxyacetic acid (2,4-D), α -Naphthaleneacetic Acid (NAA) and picloram in the suspension cultures produced somatic embryos after one week of transferring onto Murashige and Skoog (MS) solid medium without Plant Growth Regulators (PGR). With a small amount of cell inoculum (0.12 g) cultured in liquid MS medium supplemented with 2.0 mg L⁻¹ NAA for three weeks followed by one week culture on solid MS basic medium produced an average of 10.5 g fresh embryogenic cell biomass of *C. aromaticus*. The embryogenic cells differentiated to produce primordia leaves after transferred to MS medium supplemented with 1.0 mg L⁻¹ NAA.

Key words: Biomass, cell suspension cultures, *Cyperus aromaticus*, plant growth regulators, somatic embryogenesis

INTRODUCTION

Cyperus aromaticus (Ridley) Mattf and Kukenth, a perennial herb belonging to the Cyperaceae family, is grown widely in temperate and tropical region including Malaysia. It is a monocotyledonous grass-like herb with solid triquetrous culms (Nather Khan, 1990). It contains Juvenile Hormone III (JH III) with the phytochemical structure related to the JH III found in the insects (Toong *et al.*, 1988). According to Schwartz *et al.* (1998), the simple processing of the leaves of this plant may offer an effective, inexpensive and environmentally safe method of mosquito control.

Plant cell culture has been pursued for more than twenty years as a tool for the elucidation of secondary metabolites biosynthesis and as a potential means for their biotechnological production to rival extraction process based on whole plant material (Hamill and Rhodes, 1993). Somatic embryogenesis is one of the *in vitro* culture techniques by which somatic cells differentiate into somatic embryos through an orderly series of characteristic morphological stages (Vasic *et al.*, 2001; Von Arnold *et al.*, 2002). According to Martin (2003), suspension cultures induced the development of higher number of embryogenic cells. In this study, we investigate the effect of growth regulators supplemented into the culture medium on the biomass production of the embryogenic suspension cultures of *Cyperus aromaticus* to be used as the material source for the production of JH III in the future.

MATERIALS AND METHODS

Cell suspension culture of *C. aromaticus* was initiated by transferring one gram of the friable callus derived from root pieces into 100 mL Erlenmeyer flask containing 30 mL MS (Murashige and Skoog, 1962) liquid medium supplemented with 1.5 mg L⁻¹ 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 2.5 mg L⁻¹ α -Naphthaleneacetic acid (NAA), the best medium determined for callus induction. The pH of the medium was adjusted to 5.7-5.8 before autoclaving at 121°C for 13 min under a pressure of 1.05 kg cm⁻². All the cultures were placed on a rotary shaker at 100 rpm and incubated at 25±2°C under continuous light intensity of 32.5 μ E m⁻² s⁻¹. The cell suspension cultures were subcultured at 4-weeks interval. The established cell suspension cultures of *C. aromaticus* were used as the material source for subsequent studies.

Fresh weight 0.12 g of the *C. aromaticus* cells harvested from 3 weeks old cell suspension cultures were inoculated into 100 mL Erlenmeyer flask containing 30 mL MS liquid medium supplemented with one of the following Plant Growth Regulators (PGR): 2, 4-D, NAA or picloram at 0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg L⁻¹. The cultures were maintained on a gyratory shaker at 100 rpm. The flasks were placed under continuous light provided by cool white florescent lights with an intensity of 32.5 μ E m⁻² s⁻¹. The fresh weight of the cell biomass was determined from each flask. Two culture flasks were taken

randomly every week over a 4 weeks period to determine the cell biomass and the cell growth pattern. Each of the experiments was repeated three times.

The three weeks old cells cultured in MS liquid medium supplemented with 2 mg L⁻¹ NAA, MS + 2 mg L⁻¹ picloram and MS + 6 mg L⁻¹ 2, 4-D were transferred to PGR free MS solid medium for one week for the induction of embryogenic cells. These three media, MS + 2 mg L⁻¹ NAA, MS + 2 mg L⁻¹ picloram and MS + 6 mg L⁻¹ 2, 4-D, each was chosen as the best proliferation medium for *C. aromaticus* cells from each type of growth regulator. The one-week old embryogenic cells formed on the basic MS solid medium were then transferred to MS solid medium supplemented with 1 mg L⁻¹ NAA for embryos development. The biomass of the embryogenic cells were determined after one week cultured in MS basic medium. Some of the cells were removed from the embryogenic induction cultures (basic MS medium) and from the embryos development cultures (MS + 1 mg L⁻¹ NAA medium) for histological studies to determine the formation and development of the somatic embryos.

Cells for histological observation were fixed in FAA (formaldehyde: acetic acid: ethanol, 5: 5: 90) for 24 h. After dehydration process through a gradual ethanol series, these samples were embedded in xylene wax and cut into 8 µm thick slices. The samples were stained with safranin and fast green. Cultures were examined and photographed with a stereo zoom microscope (SZH, Olympus).

RESULTS AND DISCUSSION

Cell suspension cultures of *Cyperus aromaticus* were prepared by culturing the friable type of callus in MS liquid medium supplemented with three different plant growth regulators namely 2, 4-D, NAA and picloram. The cells could be maintained and showed good growth in MS liquid medium supplemented with 2.0 - 10.0 mg L⁻¹ of 2, 4-D, NAA or picloram. Basic MS medium without the addition of any growth regulators supported very low cell growth within the 4 weeks culture duration. Different type and different concentration of PGRs in the culture medium gave different effect on the production of embryogenic cell biomass of *C. aromaticus*. Maximum biomass was obtained when the cells were cultured in MS liquid medium supplemented with 2.0 mg L⁻¹ NAA (Fig. 1). When 2,4-D was supplemented into the liquid MS medium, MS + 6.0 mg L⁻¹ 2, 4-D induced the maximum cell biomass (Fig. 2). While MS liquid medium with the addition of 2.0 mg L⁻¹ picloram produced the best cell

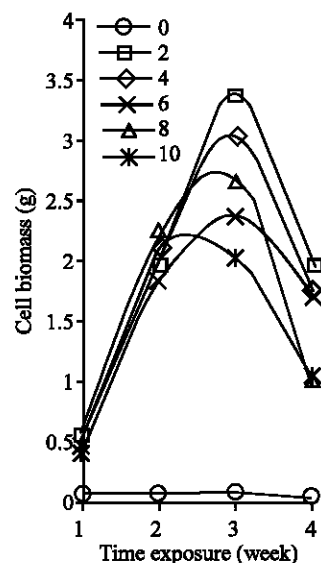


Fig. 1: Growth dynamic of *Cyperus aromaticus* suspension cultures in MS + (0-10 mg L⁻¹) NAA (n = 6)

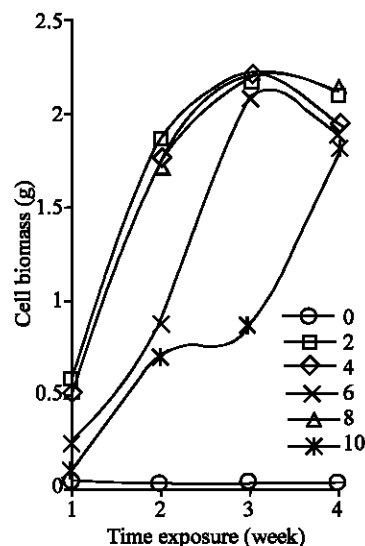


Fig. 2: Growth dynamic of *Cyperus aromaticus* suspension cultures in MS + (0-10 mg L⁻¹) 2, 4-D (n = 6)

biomass among other tested concentrations of picloram (Fig. 3). The cell biomass decreased on the 4th week for all the cells cultures growing in MS supplemented with 2, 4-D or NAA. The cells continued to grow in MS liquid medium supplemented with picloram even after 4 weeks of culture. Cells cultured in MS basic medium turned brown after one week of cultures and did not support any cell

Table 1: Formation and proliferation rate of embryogenic cells from three weeks old cells harvested from three different culture medium after transferred for one week on MS solid medium without any PGRs

Culture medium	Cell biomass in liquid medium (a) (g±sd)	Biomass of embryogenic cells after one week on MS basic medium (b) (g±sd)	Proliferation rate [b - a/a]
MS + 2.0 mg L ⁻¹ NAA	3.23±0.25	10.48±0.81	2.3
MS + 6.0 mg L ⁻¹ 2,4-D	1.91±0.27	7.00±0.53	2.7
MS + 2.0 mg L ⁻¹ picloram	2.20±0.42	8.48±0.47	3.0

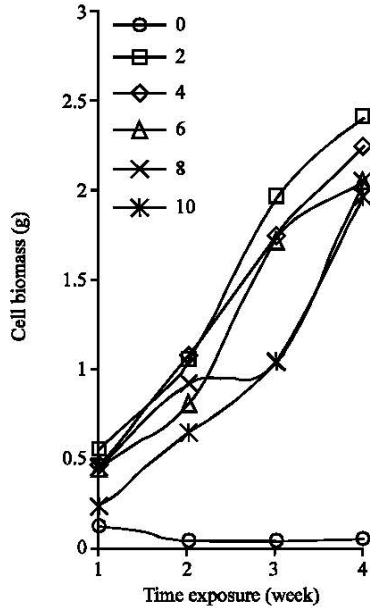


Fig. 3: Growth dynamic of *Cyperus aromaticus* suspension cultures in MS + (0-10 mg L⁻¹) picloram (n = 6)

growth subsequently (Fig. 1-3). This indicated that the cell suspension culture of *C. aromaticus* could not be maintained in the MS medium without PGRs. It was reported by Karam *et al.* (2003) that depletion of nutrient, accumulation of toxic products and absent of PGRs were other limiting factors that might have led to cell deaths and eventually to a decline in cell growth.

The cells harvested from the three weeks old cultures in MS + 2.0 mg L⁻¹ NAA, MS + 6.0 mg L⁻¹ 2, 4-D and MS + 2.0 mg L⁻¹ picloram, formed embryogenic cells after the cells were transferred onto basic MS solid medium for one week with a proliferation rate of 2.3, 2.7 and 3.0, respectively (Table 1). This showed that pretreatment of cells with different type of PGRs could affect the proliferation and formation of the somatic embryos. Vasil (1984) reported that the cultures must be plated on agar medium to obtain further development of mature somatic embryos. Tawfik and Noga (2002) and Vasic *et al.* (2001) reported that the embryogenic calli of many plant species were developed when the calli were transferred to PGR free medium. While Ruffoni and Massabü (1996) found that the embryonic cells of *Lisianthus russellianus* were



Fig. 4: Historiculture study showing the embryos of *C. aromaticus* differentiated into young primordia leaves after transferred to solid MS + 1.0 mg L⁻¹ NAA

differentiated into globular, heart and torpedo-shaped structures after 30 days in a medium without PGRs.

Present study showed that by culturing the *C. aromaticus* cells in a liquid medium as cell suspension cultures for three weeks then transferred to a solid MS medium without any growth regulators could be a very effective method for producing high quantity of cell biomass and embryogenic cells of *C. aromaticus*. With a small amount of cell inoculum (0.12 g) cultured in liquid MS medium supplemented with 2.0 mg L⁻¹ NAA for three weeks followed by one week culture on solid MS basic medium produced an average of 10.5 g fresh cell biomass of *C. aromaticus*. The pretreatment of cells with NAA was more effective compared to 2, 4-D and picloram.

Histological examination of *C. aromaticus* cells revealed that somatic embryos formed after they were transferred to PGR free solid MS medium. The embryogenesis process could be detected within the first week after cultured on PGR free MS medium. This was characterized by cells with prominent nucleus, dense cytoplasm and thicker cell wall than the normal callus cells. It was relatively isodiametric and small with respect to neighboring cells. The embryogenic cells that were transferred to 1.0 mg L⁻¹ NAA turned from light yellow to green in colour with the formation of primordia leaves

(Fig. 4). The same characteristics were reported by Quiroz-Figueroa *et al.* (2002) in their histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*.

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